INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: COILED-COIL STEM LOOP TEMPLATES

(57) Abstract

The present invention discloses polypeptides that are conformationally constricted in an intramolecular, antiparallel, coiled-coil stem loop arrangement. The coiled-coil stem comprises two α-helical structures arranged longitudinally in an antiparallel orientation such that the helices are capable of forming a superhelical structure. The loop segment comprises a connector between the carboxyl terminus of a first α-helical structure and the amino terminus of a second α-helical structure. Also disclosed are antibodies to the polypeptides of the present invention and DNA molecules which encode the polypeptides of the present invention. The present invention also relates to mimics and antagonists of biological macromolecules.

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Title

COILED-COIL STEM LOOP TEMPLATES

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Cross Reference to Related Applications

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This is a continuation-in-part of application Serial No. 08/076,088, filed
June 11, 1993.

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Field of the Invention

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The present invention relates to polypeptides that are conformationally
constricted in an intramolecular, antiparallel, coiled-coil stem loop arrangement.
The present invention also relates to mimics and antagonists of biological

15   macromolecules. The present invention further relates to antibodies directed to the
polypeptides of the present invention as well as to DNA molecules which encode the
polypeptides of the present invention.

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Background

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Recognition of specific macromolecule structures is a recurrent pattern
which underlies essentially all biological processes. For example Zinc Fingers or
repetitive zinc-binding domains (see, e.g., Miller et al., EMBO J, 4:1609-1614
(1985) and S. Harrison, Nature, 353:715-719 (1991)) are found on many

25   transcription factors which play a role in gene regulation. In essence, proteins can
be thought of as composites of conformational frameworks, or scaffolds, in which a
limited number of structural elements are incorporated onto a protein surface. For
example, commonly found macromolecular structures include β-sheets and α-
helices. The hypervariable loops of immunoglobulins are "held" in place by β-sheet

30   molecular scaffolds which are organized into a sandwich-like structure.

Another common structural motif of proteins is the α-helical bundle.
These bundles appear in different forms, including four-α-helical bundles, multiple
bundles and parallel coil-coils. Helix bundles have been reported previously by
Regan et al. (Science, 241:976-978 (1988)) and Hill et al. (Science, 249:543-546

35   (1990)). The four-helix bundle is a common folding motif found in the structure of
functionally diverse proteins such as myohemerythrin, cytochrome c' and TMV
(tobacco mosaic virus) coat protein. It may be formed from four separate
molecules, two helix-dimer molecules or one molecule containing a four-helix

construct (see, e.g., Hecht et al., Science, 249:884-891 (1990)). The arrangement of helices in naturally occurring four-helix bundles can be parallel, antiparallel or a combination thereof (see, e.g., J. Richardson, Adv. Protein Chem., 34:167 (1981)). *De novo* synthesized four-helix bundles tend to form loosely packed helices, rather

5   than compact helical structures as found in nature, and thus have limited stability in solution.

Another class of α-helical bundles is the coiled-coil, a structure adopted by the proteins keratin, myosin, epidermin, fibrinogen and tropomyosin. In nature, coiled-coil structures are frequently found on DNA binding proteins, where this

10   motif is referred to as a leucine zipper. For example, coiled-coil domains are found in the Jun, Fos (O'Shea et al., Science, 245:646-648 (1989)), C/EBP (Landschultz et al., Science, 240:1759-1764 (1988)) and GCN4 binding proteins (O'Shea et al., Science, 243:538-542 (1989)). The coiled-coil normally occurs in a parallel orientation in fibrous proteins and DNA binding proteins and is characterized by a

15   repeating seven-residue pattern. According to McLachlan and Stewart (J. Mol. Biol., 98:293 (1975)), the residue positions within the heptad repeat are termed *a, b, c, d, e, f* and *g*. A high resolution structure of the coiled-coil domain of the trans-activating protein GCN4 has recently been reported (O'Shea et al., Science, 254:539-544 (1991)). In GCN4 and other parallel coiled-coils, the residues at

20   positions *a* and *d* make side-to-side contacts with side chains *a'* and *d'*, respectively of the neighboring helix. (Primed letters refer to positions of the neighboring helix.)

The design of protein mimics and antagonists require knowledge of protein structure. If one can identify the critical structural elements on the protein surface which are responsible for interaction and function, these limited regions could be

25   excised and used to create mimics. Alternatively, small molecules which interact with these regions could be used as rationally designed antagonists. However, to design such antagonists, it is necessary to determine the three-dimensional structure of interest, to identify the structural elements required for either binding or transducing binding into function, and then design recognition molecules which

30   bind to specific structural surfaces or mimic those surfaces. The design of mimics and antagonists based on first principles of structure and function, however, is difficult to achieve due to the paucity of information of *de novo* molecular design. Even if one can achieve all of the above, small molecule mimics of proteins and ligands are often conformationally flexible and thus not of high enough affinity to

35   be a viable mimic or antagonist. A parallel coiled-coil α-helix appears to be conformationally stable and thus may bring steric order to small molecular mimics. However, formation of the coiled-coil is concentration dependent, and thus not a suitable framework for *in vivo* studies. It is thus an object of this invention to

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design mimics and antagonists of recognition macromolecules based upon a stable 2-helix, concentration-independent, coiled-coil motif.

5   Summary of the Invention

The present invention relates to a non-naturally occurring two-helix coiled-coil stem loop template molecule which comprises a single polypeptide chain comprising a first α-helical structure in an aqueous environment, a second α-helical

10   structure in an aqueous environment and a loop segment which connects the carboxyl terminus of said first α-helical structure to the amino terminus of said second α-helical structure wherein said first and second α-helical structures are arranged longitudinally in an antiparallel orientation such that said first and second helices form an intramolecular, monomeric, superhelical structure. In addition, the

15   α-helical structures may comprise heptad repeats, (abcdefg)n (SEQ ID NO: 10), of equal length, of the formula:

$$(abcdefg)n \ \overline{}\rceil$$
$$AAx$$
$$(d'c'b'a'g'f'e')n \ \underline{}\rfloor$$

wherein:

when positions a and d' are hydrophobic residues independently selected

20   from the group consisting of alanine, isoleucine, methionine and valine, then positions d and a' are leucine or when positions d and a' are hydrophobic residues independently selected from the group consisting of alanine, isoleucine, methionine and valine, then positions a and d' are leucine with the proviso that the a or d position of the first heptad repeat, which ever occurs first, and the last d' or a'

25   position of the last heptad repeat, whichever occurs last, is any amino acid residue;

e and g and e' and g' are independently selected from the group consisting of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine with the proviso that when one of two consecutive

30   heptad e, g, e' or g' positions is so selected from the group of hydrophobic-like residues consisting of valine, leucine, isoleucine, methionine, phenylalanine and tryptophan then the other heptad position may not be selected from the same group of hydrophobic-like residues, and with the additional proviso that each e and e' shall not both be positively or negatively charged and each g and g' shall not both be

35   positively or negatively charged;

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b, *c*, *f*, *b'*, *c'* and *f* are any amino acid residue with the proviso that there
cannot be more than one glycine or proline residue per heptad repeat;

n is 2-15 or fractions thereof in-between those integers; and

AAx forms a polypeptide loop segment of 4 to 15 amino acid residues. It

5   is understood that the same position within the repeating heptad units (e.g., a_1, a_2,
a_3, ...a_{15}) may or may not be the same and are selected independently of each other.

In a related aspect, the present invention relates to antibodies directed to
the polypeptides of the instant invention and further to a process to elicit antibodies
to small peptide molecules. Such process comprises inoculation of a selected

10   mammal with a coiled-coil stem loop polypeptide having the same sequence in the
loop region as the small peptide molecule of interest.

In another related aspect, this invention is a pharmaceutical composition
comprising the coiled-coil stem loop molecule as disclosed above.

In yet another related aspect, the present invention relates to an isolated

15   DNA molecule which encodes the coiled-coil stem loop molecules of the instant
invention.

In further related aspects, the present invention relates to a method to
produce helical recognition mimics which comprises synthesis of the coiled-coil
stem loop molecules of the instant invention. Such recognition sequences are

20   incorporated into the *b*, *c*, *e*, *f* and *g* positions of a heptad repeat. In addition, the
present invention relates to polypeptides produced by the method just described.

In a related aspect, the present invention relates to a method to antagonize
binding of a ligand to its receptor wherein the ligand or receptor has an α-helical
structure involved in ligand-receptor binding, which comprises binding to said

25   ligand or receptor a coiled-coil stem loop template molecule that mimics the
conformational elements of said α-helical structure.

30   Brief Description of the Drawings

FIGURE 1A. Cartoon of coiled-coil stem loop template.

FIGURE 1B. Helical wheel cross section for an antiparallel coiled-coil.
Letters *a-g* denote residue positions of an α-helical heptad repeat.

35   FIGURE 1C. Helical wheel cross section for a parallel coiled-coil. Letters
a-g denote residue positions of an α-helical heptad repeat.

FIGURE 2A. Coiled-coil stem loop template sequence (corresponds to SEQ
ID NO: 1).

FIGURE 2B. A papillomavirus E2 protein binding helix (SEQ ID NO:3) and amino acids 335-347 of the E2 protein of human papillomavirus-6 (SEQ ID NO: 2).

FIGURE 2C. An IL5-like helix (SEQ ID NO:5) and amino acids 108-126 of
5   human IL-5 (SEQ ID NO:4).

FIGURE 2D. An IL-4 binding helix (SEQ ID NO:7) and amino acids 108-126 of human IL-4 (SEQ ID NO:6).

FIGURE 3. Circular dichroism spectrum of SEQ ID NO: 1 (20 μM [micromolar]) in the presence (dashed line) and absence (solid line) of the α-helix
10   inducing solvent, trifluoroethanol (TFE). The buffer was phosphate buffered saline (PBS) and the spectra were taken at 25^0C. For the sample containing TFE, the PBS was diluted with TFE 1:1 (v/v).

FIGURE. 4. Effect of the concentration of the CCSL peptide (SEQ ID NO: 1) (also referred to as LZLS peptide) on α-helical content. The measured
15   molar ellipticity [Θ] at 222 nm is reported verses the concentration of CCSL peptide from 0.2 to 500 μM in PBS. The averaged value for the $[\Theta]_{222}$ shown by the dashed line is -28,000 deg cm^2 $dmol^{-1}$.

FIGURE 5A, B and C. Gel filtration chromatography elution profiles for the CCSL peptide (solid line) and the (VSSLESK)$_6$ peptide (SEQ ID NO:9) (dashed
20   line) loaded at a concentration of 30, 3 and 0.3 μM, respectively. The Superdex®-75 column was run at 22 °C in 150 mM sodium chloride, 20 mM phosphate, pH 7.4, (PBS) at a flow rate of 0.5 mL/min and monitoring column effluent at an absorbance of 215 nm.

FIGURE 5D. The elution profiles for the two peptides loaded at a
25   concentration of 3 μM and run in PBS containing 50% TFE (1:1,v/v).

FIGURE 6A. Selected circular dichroism spectra (CD) of the CCSL peptide taken at the indicated temperature in 150 mM sodium chloride, 20 mM phosphate, pH 7.4 (PBS).

FIGURE 6B. Thermal melting profiles of the CCSL peptide where,
30   $[\Theta]_t/[\Theta]_{5C}$, represents the molar ellipticity at 222 nm at the indicated temperature to the ellipticity at 5 °C. The inset shows the $[\Theta]_{222}/[\Theta]_{208}$ ratio for each spectra verses temperature.

FIGURE 6C. CD spectra of the CCSL peptide taken in the indicated molarity of urea in PBS.

35   FIGURE 6D. Urea denaturation profile of the CCSL peptide where, $[\Theta]_M/[\Theta]_0$, represents the fraction of ellipticity at 222 nm at the indicated molarity of urea to the ellipticity without urea. The line drawn through the data represents a nonlinear least-squares best fit assuming a two state denaturation process. The inset

shows the linear dependence of ΔG_u on the concentration of urea. The free energy of unfolding in the absence of urea is estimated by extrapolating to zero.

FIGURE 6E. Selected CD spectra of the CCSL peptide taken at the indicated pH in 150 mM sodium chloride.

5   FIGURE 6F. The pH titration curves for the CCSL peptide where, $[\Theta]_{pH9.3}$, represents the fraction of ellipticity at 222 nm at the indicated pH to the ellipticity at pH 9.3. The inset shows the $[\Theta]_{222}/[\Theta]_{208}$ ratio for each spectra verses pH.

FIGURE 7A. An IL2-like helix (SEQ ID NO:11). The dashed lines

10   indicate hydrophobic coiled-coil interface interactions. The underlined amino acids corresponds to helices A and D of human IL-2.

FIGURE 7B. Helical wheel cross section of SEQ ID NO:11.

FIGURE 8A. Gel filtration chromatography elution profiles for the IL2-like helix (SEQ ID NO:11). The elution conditions are the same as recited in
15   FIGURE 5A, B and C.

FIGURE 8B. Circular dichroism spectra (CD) of SEQ ID NO:11 taken in 150 mM sodium chloride, 20 mM phosphate, pH 7.4 (PBS).

FIGURE 9A. An IL5-like binding helix#2 (SEQ ID NO:12). The dashed lines indicate hydrophobic coiled-coil interface interactions. The underlined amino
20   acids corresponds to helices A and D of human IL-5.

FIGURE 9B. Helical wheel cross section of SEQ ID NO:12.

FIGURE 10A. Gel filtration chromatography elution profiles for the IL5-like helix#2 (SEQ ID NO:12). The elution conditions are the same as recited in FIGURES 5A, B and C.
25   FIGURE 10B. Circular dichroism spectra (CD) of SEQ ID NO:12 taken in 150 mM sodium chloride, 20 mM phosphate, pH 7.4 (PBS).

Detailed Description

The present invention comprises polypeptides that are conformationally
constricted and are thus useful as carriers or scaffolds to present amino acid
sequences of interest in a particular three dimensional conformation. The molecules
of the invention assume the following conformation in an aqueous environment: an
antiparallel, coiled-coil α-helical stem and an internal loop, collectively and
hereinafter referred to as a coiled-coil stem loop (CCSL) or a coiled-coil recognition
molecule. The stem comprises an intramolecular heterodimer of two α-helical
sequences in an antiparallel orientation such that said helices are capable of forming
an intramolecular, and monomeric, superhelical structure, for example, like an
antiparallel leucine zipper. The internal loop or loop segment connects the carboxy
terminus of a first α-helical structure to the amino terminus of a second α-helical
structure. Such a coiled-coil stem loop is depicted in Figure 1.

In general, a coiled-coil arrangement is thought to be conformationally
ordered and thus useful for bringing steric constraint to small molecular mimics.
The stability of such intermolecular coiled-coils is, however, dependent on peptide
concentration. That is, two separate α-helices are in equilibrium with a dimeric or
superhelical structure, and thus dimerization can occur only in high concentration of
the individual α-helices, i.e., the monomers. At low monomeric concentration, the
dimeric structure dissociates. Thus, such intermolecular coiled-coils are of limited
use in an *in vivo* application. A significant advantage of the instant invention is that
it eliminates this concentration dependence. The molecules of the present invention
comprise intramolecular antiparallel coiled-coils, which are more stable than
intermolecular coiled-coils because the latter are concentration dependent. Another
advantage of the present invention is that the coiled-coil stem loop structure forms
spontaneously in an aqueous environment to form a monomeric, intramolecular
superhelical structure. Confirmation of monomeric (verses dimeric) structure can be
readily determined by techniques known in the art, e.g., gel filtration
chromatography, equilibrium sedimentation centrifugation, NMR, etc.

Recognition elements which can be mimicked in the coiled-coil stem loop
include α-helical surfaces, continuous loop residues and chemical groups brought
together in space by specific placement due to the coiled-coil and loop domains.
The coiled-coil stem loop of the present invention provides a template for molecular
recognition elements. For example, the CCSL can be adapted to form specific
recognition molecules (i.e., helical recognition mimics) by presenting helical
recognition sequences from naturally occurring proteins. Specific examples of

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helices for which mimicry can be tested with the coiled coil stem loop include, but
are not limited to: papillomavirus E2 protein, a DNA-binding transcriptional
regulator; interleukin 5, a cytokine which stimulates eosinophil maturation,
proliferation and activation; and interleukin 4, a cytokine which activates resting B

5   lymphocytes and is involved in immunoglobulin class switching. It is appreciated to
one of skill in the art that the helical surfaces from naturally occurring proteins can
be grafted onto either one or both of the coiled-coil helical sequences to form
monovalent or bivalent mimics. The helical recognition mimics produced by the
present invention can be used to antagonize the binding site of a ligand (e.g., cell

10   surface receptors), provided the binding site interacts with an α-helical structure
which has been mimicked. The present invention thus relates to a method to
produce helical recognition mimics which comprises synthesis of the coiled-coil
stem loop molecules of the present invention wherein the recognition sequences are
preferentially (but not always) incorporated into the b, c, e, f and g positions and/or

15   the b', c', e', f' and g' positions of a heptad repeat. In addition, the present invention
relates to polypeptides produced by said method or process. Furthermore, the
present invention relates to a method to antagonize binding of a ligand to its
receptor wherein the ligand or receptor has an α-helical structure involved in ligand-
receptor binding, which comprises binding to said ligand or receptor a coiled-coil

20   stem loop template molecule that mimics the conformational elements of said α-
helical structure.

        The coiled coil stem loop can also be adapted by presenting recognition
sequences into the loop segment. For example, the RGD (Arginine-Glycine-
Aspartic Acid) sequence known to bind the fibrinogen receptor (i.e., a ligand) has

25   been placed in the loop of a coiled-coil stem loop (CCSL) as disclosed in Figure 2A
(SEQ ID NO: 1). This molecule has the ability to bind fibrinogen receptor with an
affinity at least as great as that of RGD containing linear peptides. Another
approach is to mimic the binding site of a receptor. For example, residues Gln^{40} to
Phe^{43} and/or residues Val^{86} to Gln^{89} of the CD4 receptor protein could be placed in

30   the loop region of the (CCSL) stem loop to test inhibition of ligand binding (e.g.,
HIV envelope protein) to said receptor. In addition, the sequence composition and
length of the loop can be varied to form a diverse set of molecules which may bind
to macromolecular receptors with various affinities due to differences in
composition and conformation.

35   The α-helical structures of the present invention comprise a plurality of
heptad repeats, of which both α-helical structures are of equal length. According to
McLachlan and Stewart (J. Mol. Biol., 98:293 (1975)), the positions within the

heptad repeat are termed a, b, c, d, e, f and g. Hence the α-helical structures can be represented by the formula:

$$(abcdefg)n \quad \text{(SEQ ID NO: 10)}$$

It is appreciated, however, that the heptad repeat may start at any position, e.g.,
5   (bcdefga)n or (cdefgab)n, etc.

Each heptad repeat is a right handed α-helix. The geometry of each heptad in a coiled-coil conformation is a repeating unit where every seventh residue of an amino acid sequence is at a structurally equivalent position. For example, the a position always appears on the same hydrophilic interior surface of the α-helix. The
10   first and second α-helical structures interact longitudinally in an antiparallel orientation such that they are capable of forming an intramolecular superhelical structure under a range of conditions (see, e.g., the Examples section). Such structure is akin to strands of rope which form a central superhelical axis. Thus for a superhelical structure, each of the helices is slightly distorted from the central axis
15   and the overall superhelix has a left handed twist.

The positions a, b, c, d, e, f and g within the heptad repeat can be categorized into three groups based upon helical geometry. Ideally the profile comprises a hydrophilic exterior, a hydrophobic interior and a border of polar residues, preferably with charged side chains (i.e., R groups) to form interhelical salt
20   bridging residues. The positions of the first α-helical structure are designated a, b, c, d, e, f and g and the positions of the second, or antiparallel, α-helical structurere are designed with " ' ", e.g., a', b', c', d', e', f' and g'.

The coiled-coil stem loop molecules of the invention can be depicted as follows:

$$(abcdefg)n \quad \overline{}\rceil$$
$$\text{AAx}$$
25     $$(d'c'b'a'g'f'e')n \quad \underline{}\rfloor$$

It is important to note that in an antiparallel α-helical coiled-coil, the side chains of residues a pack against the side chain residues of position d', while the side chains of residues at position d pack against the side chains of residues a', where
30   primed letters refer to positions of the neighboring helix. For example, if n were to be assigned the value of 1, a_1 would interact with d'_1 and d_1 would interact with a'_1. If n were assigned the value of 2:

$$(a_1, b_1, c_1, d_1, e_1, f_1, g_1)(a_2, b_2, c_2, d_2, e_2, f_2, g_2) \quad \overline{}\rceil$$

$$\text{loop segment}$$
35     $$(d'_2,c'_2,b'_2,a'_2,g'_2f'_2,e'_2)(d'_1,c'_1,b'_1,a'_1,g'_1f'_1,e'_1) \quad \underline{}\rfloor$$
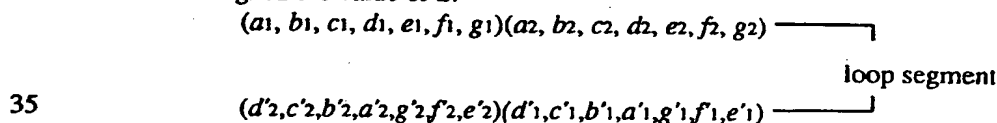
a_1 would interact with d_2, d_1 would interact with a_2, a_2 would interact with d_1 and d_2 would interact with a_1. Similar interactions can be envisioned when n = 3 to 15.

Positions a and d are typically hydrophobic residues, forming the hydrophobic interface of the coiled-coil and are believed to stabilize antiparallel
5   helix dimerization in the present invention. Such hydrophobic residues comprise alanine, valine, leucine, isoleucine, methionine, phenylalanine, and tryptophan as well as modifications to naturally occurring amino acids which are known in the art. Preferably, the hydrophobic residues are selected from the group consisting of leucine, isoleucine, methionine, valine and alanine with the proviso that at least one
10 residue is leucine. More preferably, when positions a and d' are hydrophobic residues independently selected from the group consisting of alanine, isoleucine, methionine and valine, then positions d and a' are leucine and similarly, when positions d and a' are hydrophobic residues independently selected from the group consisting of alanine, isoleucine, methionine and valine, then positions a and d' are
15 leucine with the proviso that the a or d position of the first heptad repeat, which ever occurs first, and the last d' or a' position of the last heptad repeat, whichever occurs last, is any amino acid residue. Preferably, a or d' (or d and a', respectively) are the same amino acid residue. It is still more preferable that when a or d' (or d and a') is leucine, then the other position (i.e., d or a', or a or d', respectively) is alanine,
20 isoleucine or valine, more prefereably alanine or valine and most preferably valine.

Positions e and g and e' and g' are independently selected from the group consisting of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine with the proviso that when one of
25 two consecutive heptad positions (e.g., e_1, g_1, e'_1, etc.) is so selected from the group of hydrophobic-like residues consisting of valine, leucine, isoleucine, methionine, phenylalanine and tryptophan then the other structurally equivalent heptad position (e.g., e_2, g_2, e'_2, etc.) may not be selected from the same group of hydrophobic-like residues, and with the additional proviso that each e, and the antiparallel equivalent
30 e' (e.g., e_1 and e'_1), shall not both be positively or negatively charged, and each g, and the antiparallel equivalent g', shall not both be positively or negatively charged. More preferably, e and g and e' and g' are selected from the group consisting of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, histidine, lysine, serine, threonine and tyrosine. More preferably yet, e and g are
35 charged residues 50% or greater of the time which facilitate interhelical ionic or electrostatic interactions, for example, residues which form interhelical salt bridges. Such residues may comprise aspartic acid, glutamic acid, lysine, arginine, histidine and other residues which can be modified to have an overall net charge (e.g.,

tyrosine residues can be modified at the hydroxyl group to form carboxylic acids, amines, etc.). It is preferable that the overall net charge of one helix (i.e., all e and g residues of the first α-helical structure) is opposite to that of the second α-helical structure (i.e., all e' and g' residues) and that the residues at one position (e.g., e_1)

5   which interact with a corresponding position of the coiled-coil stem loop (e.g., e'_1 (or g_1 and g'_1)) do not have the same charge. For example, if the residue at position e_1 contained a positively charged group, then e'_1 would preferably be negatively charged or uncharged. It is most preferable that positions e, g, e' and g' comprise arginine, aspartic acid, lysine and glutamic acid, such that a positively charged

10   residue (e.g., lysine) on one α-helix will interact with a negatively charged residue (e.g., glutamic acid) on the second (or antiparallel) α-helical structure and vice versa.

Positions b, c, f, b', c' and f' are less important for intramolecular dimerization of the α-helices than the other positions. They are located on the

15   exterior surface on the coiled-coil and are exposed to the aqueous environment. Residues at these positions may be used to stabilize an α-helix, or be used as recognition surfaces to form α-helical mimics. The residues at positions b, c, f, b', c' and f' can be virtually any amino acid with the proviso that there cannot be more than one glycine or proline residue per heptad repeat. Preferably, b, c, f, b', c' and f'

20   are comprised of 25% or less of "strongly hydrophobic" residues (i.e., leucine, isoleucine, glycine, methionine, phenylalanine, tryptophan and valine). More preferably, b, c, f, b', c' and f' are independently selected from the group consisting of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, lysine, serine, threonine and tyrosine. When the residues at positions b, c, f, b', c'

25   and f' are not intended to be used as recognition surfaces for α-helical mimics, then it is preferable that those positions are selected from the group consisting of alanine, serine and threonine.

n is 2 - 15 or fractions (i.e., sevenths, for example 1/7, 2/7, 3/7, etc.) thereof in-between the integers 2 - 15. Preferably, n is 3 to 10 repeating units or fractions

30   thereof. More preferably, n is 3 to 7 repeating units and fractions thereof (e.g., n can be 3 4/7). It is understood that the same position within the repeating heptad units (e.g., a_1, a_2, a_3, ... a_{15}) may or may not be the same and is selected independently from each other.

The coiled-coil motif is highly tolerant of amino acid substitutions so long

35   as the α-helix is not substantially destabilized. For a discussion of alpha helix formers and breakers, see Chou and Fasman (Biochem., 13:211-222 (1974)).

In addition to the α-helical structures, the present invention comprises a loop segment (AAx) which connects the two α-helical structures. The first and last

residues (in a N-terminus to C-terminus orientation) of the non-helical region can be any amino acid. Preferably, one or both residues are helix-breaking or helix destabilizing residues (see, Chou and Fasman, *supra*), for example, glycine and proline. The non-helical region or loop segment comprises from about 4 to 15

5   amino acid residues. Preferably the non-helical region comprises from about 5 to 10 amino acid residues.

In addition to these general considerations for the formation of α-helices of this invention, one can further promote helix formation and stability in the molecules of this invention by including negatively charged groups at the N-

10   terminal end and positively charged groups at the C-terminal end of the coiled-coil stem loop molecule to stabilize the dipole (see, e.g. Fairman et al., Proteins: Structure, Function and Genetics, 5:1-7 (1989)). Alternatively, one can block the charged amino and carboxyl groups of the first and last residues (i.e., NH_3^+ and COO^-) with, for example, an acetyl group and an amide group, respectively. The

15   effect of blocking these charged groups is to decrease the dipole moment across the peptide bonds along the helix, thus promoting helix stability (see., e.g., Shoemaker et al., Nature, 236:563-567 (1987)).

Another method to further stabilize the overall coiled-coil structure is to design coiled-coil stem loop molecules with residues at the amino terminus and

20   carboxy terminus that are capable of forming covalent bonds with each other. For example, disulfide bonds or homodetic peptide cyclization.

The coiled-coil stem loops of this invention comprise non-naturally occurring two-helix sequences which may optionally mimic residues of naturally occurring polypeptides. (See, e.g. Figure 2). The CCSL template molecules may be

25   synthesized by a variety of means, for example, by recombinant DNA technology or by chemical synthesis. Methods of well-known peptide synthesis are generally set forth by Ali et al., J. Med. Chem., 29:984 (1986) and J. Med. Chem., 30:2291 (1987) and are incorporated by reference herein. Preferably, the peptides are prepared by the solid phase technique of Merrifield (J Am Chem Soc, 85:2149

30   (1964)). However, a combination of solid phase and solution synthesis may be used, as in a convergent synthesis in which di-, tri-, tetra-, or penta-peptide fragments may be prepared by solid phase synthesis and either coupled or further modified by solution synthesis.

During synthesis, the side chain functional groups (e.g., $-NH_2$, $-COOH$, $-OH$,

35      $-SH$) are protected during the coupling reactions. Normally, the α-amino group is temporarily protected as t-Butoxycarbonyl (BOC) but other acid or base labile protecting groups can be used, e.g., fluorenylmethoxycarbonyl (FMOC). The amino side chain group of lysine is protected as benzyloxycarbonyl or p-

chlorobenzyloxycarbonyl (Z or Cl-Z). Para-methylbenzyl (p-MBz) or
acetomidomethyl protection is used for cysteines. Hydroxy groups are protected as
benzyl ethers and carboxyl groups are protected as benzyl (Bz) or cyclohexyl esters.

5   The peptides of the present invention can be synthesized either from the C-
terminus or the N-terminus. Preferably it is from the C-terminus. Prior to coupling
the alpha-carboxyl group (of a suitable protected amino acid) is activated. One
skilled in the art can activate the protected group in a number of ways. For example,
one may use N,N' dicyclohexylcarbodiimide (DCC), p-nitrophenyl esters (pNp),
hydroxybenzotriazole ester (HOBt), N-hydroxy succinimidyl ester (Osu) mixed
10   anhydride or symmetrical anhydride.

Solution synthesis of peptides is accomplished using conventional methods to
form amide bonds. Typically, a protected Boc-amino acid which has a free carboxyl
group is coupled to a protected amino acid which has a free amino group using a
suitable carbodiimide coupling agent, such as N, N' dicyclohexyl carbodiimide
15   (DCC), optionally in the presence of a catalyst such as 1-hydroxybenzotriazole
(HOBt) and dimethylamino pyridine (DMAP).

In solution phase synthesis, the coupling reactions are preferably carried out
at low temperature (e.g., -20°C) in such solvents as dichloromethane (DCM),
dimethyl formamide (DMF), N-methyl pyrrolidone (NMP), tetrahydrofuran (THF)
20   acetonitrile (ACN) or dioxane.

If solid phase methods are used, the peptide is built up sequentially starting
from the carboxy terminus and working towards the amino terminus of the peptide.
Solid phase synthesis begins by covalently attaching the C terminus of a protected
amino acid to a suitable resin, such as methyl benzhydrylamine (mBHA).
25   In solid phase synthesis, the first amino acid residue is normally attached to
an insoluble polymer. For example, two commonly used polymers are polystyrene
(1% cross-linked with divinyl benzene) and 1% cross-linked polyamides. These
polymers are functionalized to contain a reactive group, e.g., -OH, -NH$_2$ and -
CH$_2$Cl to link the first amino acid of the targeted peptide (i.e., carboxy terminus).
30   The choice of the linkage between the first amino acid and the polymer is dictated
by the carboxy terminus of the peptide. For example, peptides having a carboxyl
group at the C-terminus would be linked by an ester linkage and peptides with a
carboxamide ending would have an amide linkage.

Once the first protected amino acid has been coupled to the desired resin, the
35   protected amino group is hydrolyzed by mild acid treatment, and the free carboxyl
of the next (protected) amino acid is coupled to this amino group. This process is
carried out sequentially, without isolation of the intermediate, until the peptide of

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interest has been formed. The completed peptide may then be deblocked and/or cleaved from the resin in any order.

Preferred solvents for the coupling reactions include, but are not limited to, dichloromethane (DCM), dimethyl formamide (DMF) and N-methyl pyrrolidone

5   (NMP). After the desired sequence is synthesized, the peptide is deprotected and cleaved from the resin using standard techniques (e.g., using hydrofluoric acid (HF)).

The preferred method for cleaving a peptide from the support resin is to treat the resin-supported peptide with anhydrous HF in the presence of a suitable cation

10   scavenger, such as anisole or dimethoxy benzene.

To obtain the conformationally restricted peptides of the present invention, the synthetic peptide may be cyclized using methods well known in the art. For example, there are numerous protocols for forming intramolecular bonds between cysteine residues. In performing these reactions, water, methanol, acetic acid, DMF

15   or a suitable mixture of these solvents can be used.

Formation of the disulfide bond may be accomplished by several known methods. If the sulfur-containing amino acids of the linear peptide are protected differently, in such a manner as to allow formation of a mono mercaptan, cyclization may be effected by base catalyzed nucleophilic displacement of the protecting group

20   of the sulfur-containing amino acid. Groups which are especially useful as displaceable protecting groups are thioalkyl or thioaryl groups. Exemplary of this method is the protection of one sulfur-containing amino acid by the thioethyl group, and protection of the second by a substituted benzyl group. Deprotection of such a peptide by HF removes the benzyl group from one amino acid, while leaving the

25   second protected as an ethyl disulfide. Stirring this mercaptoβdisulfide in dilute solution at a pH of about 7 to 8 effects displacement of the thioethyl group and cyclization of the linear peptide. When cysteine is protected with an ACM (acetamido methyl) group the preferred method is to oxidize with iodine.

If the corresponding linear peptide is completely deprotected and produced as

30   a dimercaptan, any oxidizing agent known in the art to be capable of converting a dimercaptan to a disulfide may be used. Exemplary of such agents are an alkali metal ferricyanide, (e.g., potassium or sodium ferricyanide), oxygen gas, diiodomethane or iodine. The reaction is conducted in a suitable inert solvent, such as aqueous methanol or water, at temperatures from about 0 to 40°C, under high

35   dilution. The pH is usually maintained at about 7 to 8. Cyclization may be performed upon the peptide while it is still attached to the support resin or while other functional groups are still protected, but it is preferably performed on the deprotected free peptide.

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Acid addition salts of the peptides are prepared in a standard manner in a suitable solvent from the parent compound and an excess of an acid, such as hydrochloric, hybrobromic, sulfuric, phosphoric, acetic, maleic, succinic, or methanesulfonic. The acetate salt form is especially useful. Certain of the

5   compounds form inner salts or zwitterions which may be acceptable. Cationic salts are prepared by treating the parent compound with an excess of an alkaline reagent, such as a hydroxide, carbonate or alkoxide containing the appropriate cation. Cations such as Na^+, K^+, Ca^{2+} and NH_4^+ are examples of cations present in pharmaceutically acceptable salts.

10   The peptides can then be purified by a number of techniques. Preferred embodiments include reverse phase HPLC, counter current distribution (CCD) and crystallization. More preferably, HPLC is used. The purified products can then be analyzed for purity using HPLC, amino acid analysis and fast atom bombardment mass spectrometry (FAB-MS).

15   Alternatively, coding sequences for the polypeptides of the present invention can be recombinant DNA molecules which are introduced into expression vectors or phage (collectively referred to as "vectors") by operatively linking the DNA to the necessary expression control regions (e.g. regulatory regions) required for gene expression. The vectors can be introduced into the appropriate host cells such as

20   prokaryotic (e.g., bacterial), or eukaryotic (e.g., yeast or mammalian) cells by methods well known in the art (see, e.g., "Current Protocol in Molecular Biology", Ausubel et al. (eds.), Greene Publishing Assoc. and John Wiley Interscience, New York, 1989 and 1992). Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice.

25   Examples of recombinant DNA vectors for cloning and host cells which they can transform include, but are not limited to, the bacteriophage λ (E. coli), pBR322 (E. coli), pACYC177 (E. coli), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-E. coli gram-negative bacteria), pHV14 (E. coli and Bacillus subtilis), pBD9 (Bacillus), pIJ61

30   (Streptomyces), pUC6 (Streptomyces), YIp5 (Saccharomyces), pAcYM1 (baculovirus insect cell system) and YCp19 (Saccharomyces). See, also, "DNA Cloning": Vols. I & II, Glover et al. ed. IRL Press Oxford (1985) (1987) and; T. Maniatis et al. "Molecular Cloning" Cold Spring Harbor Laboratory (1982).

The gene can be placed under the control of a promoter, ribosome binding

35   site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the desired protein is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal

peptide or leader sequence. The subunit antigens of the present invention can be expressed using, for example, the E. coli tac promoter or the protein A gene (spa) promoter and signal sequence. Leader sequences can be removed by the bacterial host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739;
5   4,425,437; 4,338,397.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to
10   be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and
15   orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding the particular antigen of interest may be desirable to achieve this end. For example, in
20   some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an
25   expression vector which already contains the control sequences and an appropriate restriction site.

In some cases, it may be desirable to add sequences which cause the secretion of the polypeptide from the host organism, with subsequent cleavage of the secretory signal. It may also be desirable to produce mutants or analogs of the receptors of
30   interest. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. See, e.g., T. Maniatis et al., supra; DNA Cloning, Vols. I and II, supra; Nucleic
35   Acid Hybridization, supra.

A number of prokaryotic expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,578,355; 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832; see also U.K. Patent

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Applications GB 2,121,054; GB 2,008,123; GB 2,007,675; and European Patent
Application 103,395. Yeast expression vectors are also known in the art. See, e.g.,
U.S. Patent Nos. 4,446,235; 4,443,539; 4,430,428; see also European Patent
Applications 103,409; 100,561; 96,491. pSV2neo (as described in J. Mol. Appl.

5   Genet. 1:327-341) which uses the SV40 late promoter to drive expression in
mammalian cells or pCDNA1neo, a vector derived from pCDNA1 (Mol. Cell Biol.
7:4125-29) which uses the CMV promoter to drive expression. Both these latter two
vectors can be employed for transient or stable (using G418 resistance) expression in
mammalian cells. Insect cell expression systems, e.g., Drosophila, are also useful,

10   see for example, PCT applications US 89/05155 and US 91/06838 as well as EP
application 88/304093.3.

Depending on the expression system and host selected, the proteins of the
present invention are produced by growing host cells transformed by an expression
vector described above under conditions whereby the protein of interest is expressed.

15   The protein is then isolated from the host cells and purified. If the expression
system secretes the protein into growth media, the protein can be purified directly
from the media. If the protein is not secreted, it is isolated from cell lysates or
recovered from the cell membrane fraction. The selection of the appropriate growth
conditions and recovery methods are within the skill of the art.

20   Confirmation of α-helical formation can be accomplished by known
techniques in the art, for example, by circular dichroism (CD) monitoring,
fluorescence spectra monitoring, NMR, thermal denaturation and x-ray diffraction
analysis.

Once a suitable scaffold is designed, the recognition peptide sequences can

25   be inserted into it and the scaffolding or conformational framework is used to limit
flexibility. The sequence in or adjacent to the recognition peptide could be mutated
and sufficiently high-affinity species selected by an affinity capture method. For
example, filamentous phage surface proteins (see, e.g., Scott et al., Science, 249:386
(1990) and Devlin et al., Science, 249:404 (1990) which are hereby incorporated by

30   reference) could be used for random mutagenesis and then select for high-affinity
peptide sequences by a suitable affinity screen/selection method. The invention can
thus be used to identify amino acid residues which bind to macromolecules,
including receptors. Such compositions can antagonize the interactions of the
biological macromolecules and thereby antagonize their biological activities.

35   Coiled-coil stem loops which function as antagonists can be used directly
as therapeutic agents. For example, the CCSL may be an inhibitor (or modulator) of
proteins, e.g., anti-inflammatory agents which neutralize cell adhesion molecules,
cytokines, etc. The CCSL can also be modified by selective changes (e.g.,

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chemically or by site directed mutagenesis, i.e., insertion, deletion, substitution or
rearrangements) to modulate macromolecular affinity and specificity.

     A coiled-coil stem loop with antagonist activity also provides a chemical
lead for SAR (structure activity relationships) and xenobiotic design. The structure

5   of an active coiled-coil stem loop can be predicted or determined experimentally.
The structure defines the organization of binding elements. These elements can be
reconstituted into small organic molecules by synthetic chemistry approaches.

     In addition, a coiled-coil stem loop which mimics the binding site of a
biological macromolecule can be used as an immunogen to produce neutralizing

10   antibodies for the activity of that molecule. Alternatively, the coiled coil stem loop
can be used to stimulate or elicit an immunogenic response (e.g., as a vaccine), by
presenting an antigenic site in a configuration which stimulates production of
protective antibodies. For example, a small peptide sequence (i.e., less than 50
residues, preferably less than 15 residues, more preferably less than 10 residues) can

15   be duplicated or found in the non-helical loop region of the CCSL which is then
used to inoculate a selected mammal.

     Thus proteins encompassed by the present invention or fragments thereof
comprising at least one epitope can be used to elicit or produce antibodies, both
polyclonal and monoclonal. If polyclonal antibodies are desired, a selected

20   mammal, (e.g., mouse, rabbit, rat, goat, horse, primate, human, etc., preferably
mouse, rat or rabbit) is immunized with a CCSL template molecule of the present
invention. Serum from the immunized animal is collected and treated according to
known procedures. If serum containing polyclonal antibodies is used, the polyclonal
antibodies can be purified by immunoaffinity chromatography or other known

25   procedures.

     Monoclonal antibodies to the proteins of the present invention, and to the
fragments thereof, can also be readily produced by one skilled in the art. The
general methodology for making monoclonal antibodies by using hybridoma
technology is well known. Immortal antibody-producing cell lines can be created by

30   cell fusion, and also by other techniques such as direct transformation of B
lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See,
e.g., M. Schreier et al., "Hybridoma Techniques" (1980); Hammerling et al.,
"Monoclonal Antibodies and T-cell Hybridomas" (1981); Kennett et al.,
"Monoclonal Antibodies" (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121;

35   4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500; 4,491,632; and 4,493,890.
Panels of monoclonal antibodies produced against the antigen of interest, or
fragment thereof, can be screened for various properties; i.e., for isotype, epitope,
affinity, etc. Monoclonal antibodies are useful in purification, using immunoaffinity

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techniques, of the individual antigens which they are directed against. Alternatively, genes encoding the monoclonals of interest may be isolated from the hybridomas by PCR techniques known in the art and cloned and expressed in the appropriate vectors. The antibodies of this invention, whether polyclonal or monoclonal have

5   additional utility in that they may be employed reagents in immunoassays, RIA, ELISA, and the like. As used herein, "monoclonal antibody" is understood to include antibodies derived from one species (e.g., murine, rabbit, goat, rat, human, etc.) as well as antibodies derived from two (or perhaps more) species (e.g., chimeric and humanized antibodies).

10   Chimeric antibodies, in which non-human variable regions are joined or fused to human constant regions (see, e.g. Liu et al., Proc. Natl Acad. Sci. USA, 84:3439 (1987)), may also be used in assays or therapeutically. Preferably, a therapeutic monoclonal antibody would be "humanized" as described in Jones et al., Nature, 321:522 (1986); Verhoeyen et al., Science, 239:1534 (1988); Kabat et al., J.

15   Immunol., 147:1709 (1991); Queen et al., Proc. Natl Acad. Sci. USA, 86:10029 (1989); Gorman et al., Proc. Natl Acad. Sci. USA, 88:34181 (1991); and Hodgson et al., Bio/Technology, 9:421 (1991).

            Pharmaceutical compositions of the polypeptides prepared as hereinbefore described and other peptide or polypeptide derivatives may be formulated as

20   solutions of lyophilized powders for parenteral administration. Powders may be reconstituted by addition of a suitable diluent or other pharmaceutically acceptable carrier prior to use. The liquid formulation is generally a buffered, isotonic, aqueous solution. Examples of suitable diluents are normal isotonic saline solution, standard 5% dextrose in water or buffered sodium or ammonium acetate solution.

25   Such formulation is especially suitable for parenteral administration, but may also be used for oral administration or contained in a metered dose inhaler or nebulizer. It may be desirable to add excipients such as polyvinylpyrrolidone, gelatin, hydroxy cellulose, acacia, polyethylene glycol, mannitol, sodium chloride or sodium citrate. Actual methods for preparing parenterally administrable compositions are well

30   known or will be apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania.

            The compounds described herein can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be

35   effective with conventional proteins and art-known lyophilization and reconstitution techniques can be employed.

            Alternatively, these peptides may be encapsulated, tableted or prepared in a emulsion or syrup for oral administration. Pharmaceutically acceptable solid or

liquid carriers may be added to enhance or stabilize the composition, or to facilitate preparation of the composition. Solid carriers include starch, lactose, calcium sulfate dihydrate, terra alba, magnesium stearate or stearic acid, talc, pectin, acacia, agar or gelatin. Liquid carriers include syrup, peanut oil, olive oil, saline and water.

5   The carrier may also include a sustained release material such as glyceryl monostearate or glyceryl distearate, alone or with a wax. The amount of solid carrier varies but, preferably, will be between about 20mg to about 1g per dosage unit. The pharmaceutical preparations are made following the conventional techniques of pharmacy involving milling, mixing, granulating, and compressing,

10   when necessary, for tablet forms; or milling, mixing and filling for hard gelatin capsule forms. When a liquid carrier is used, the preparation will be in the form of a syrup, elixir, emulsion or an aqueous or non-aqueous suspension. Such a liquid formulation may be administered directly or filled into a soft gelatin capsule.

       For rectal administration, the peptides of this invention may also be
15   combined with excipients such as cocoa butter, glycerin, gelatin or polyethylene glycols and molded into a suppository.

       The physician will determine the dosage of the present therapeutic agents which will be most suitable and it will vary with the form of administration and the particular compound chosen, and furthermore, it will vary with the particular patient
20   under patient under treatment. He will generally wish to initiate treatment with small dosages substantially less than the optimum dose of the compound and increase the dosage by small increments until the optimum effect under the circumstances is reached. It will generally be found that when the composition is administered orally, larger quantities of the active agent will be required to produce
25   the same effect as a smaller quantity given parenterally. The compounds are useful in the same manner as other serotonergic agents and the dosage level is of the same order of magnitude as is generally employed with these other therapeutic agents. The therapeutic dosage will generally be from 1 to 10 milligrams per day and higher although it may be administered in several different dosage units. Tablets
30   containing from 0.5 to 10 mg. of active agent are particularly useful.

       Depending on the patient condition, the pharmaceutical composition of the invention can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient already suffering from a disease in an amount sufficient to cure or at least partially arrest the disease
35   and its complications. In prophylactic applications, compositions containing the present compounds or a cocktail thereof are administered to a patient not already in a disease state to enhance the patient's resistance.

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Single or multiple administrations of the pharmaceutical compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical composition of the invention should provide a quantity of the compounds of the invention sufficient to effectively treat the patient.

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The examples which follow are illustrative, and not to be construed as limiting of the present invention.

EXAMPLES

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Materials. Solid phase peptide synthesis resins were obtained from Applied Biosystems, Inc. (Foster City, CA). Protected amino acids were purchased from Bachem (Philadelphia, PA), Keystone Bio-Tec (Philadelphia, PA), and Peninsula Laboratories (Belmont, CA). Diisopropylcarbodiimide was obtained
15 from Milligen/Biosearch (Burlington, MA). Diisopropyethylamine, hydroxybenzotriazole, 1-methyl-2-pyrrolidinene, anisole, dimethyl sulfide, *m*-cresol, ethanedithiol, piperidine, pyridine, and buffer salts were obtained from Aldrich (Milwaukee, WI). All solvents were HPLC grade and were purchased from Baker (Philipsburg, NJ) or Mallinekrodt, Inc. (Paris, Kentucky). Guanidinium
20 hydrochloride, 2-mercaptoethanol, and urea were obtained from Pierce (Rockford, Illinois). 1000 MW cutoff dialysis membrane was obtained from Spectrum (Los Angeles, CA). HPLC columns were from Vydec (Hesperia, CA), Bio-Rad (Richmond, CA), Rainin (Woburn, MA), or Beckman (Palo Alto, CA).

25   Peptide Synthesis of CCSL: The CCSL peptide (SEQ ID NO: 1) was synthesized by solid phase peptide synthesis carried out using the standard t-butoxycarbonyl (tBoc) strategy on a Beckman 990 automated peptide synthesizer. The starting resin was p-methylbenzhyldrylamine (MBHA) and the amino acid derivatives employed included: Boc-Leu, Boc-Ala, Boc-Glu(oBzl), Boc-Ser(Bzl),
30 Boc-Val, Boc-Gly, Boc-Arg(Tos), Boc-Asp(Chx), Boc-Met, Boc-Pro, Boc-Lys(Z), and Boc-cys(Acm). The blocking groups were removed by HF cleavage in the presence of anhydrous anisole. The peptides GRGDMP (SEQ ID NO:8), (VSSLESK)$_6$[1] (SEQ ID NO: 9), IL2-like Helix (SEQ ID NO:11) and IL5-like Helix#2 (SEQ ID NO:12) were synthesized by similar techniques and were purified
35 by preparative HPLC using delta-pack C18 from Millipore Co. Peptide purity was verified to be greater than 90% by amino acid composition analysis (Beckman 6300

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[1]This peptide forms a homodimer or an intermolecular coiled-coil arrangement.

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Gold) and analytical C18 reversed-phase HPLC (5μ from Vydac separation groups).
Fast atom bombardment mass spectrometry for the CCSL peptide gave a molecular
weight M_r of 5,790.0 (calculated M_r=5,789.4).

5   Gel Filtration Chromatography Gel filtration chromatography was
performed on a 30/16 Superdex®-75 column (Pharmacia) in 150 mM sodium
chloride, 20 mM phosphate, pH 7.4, (PBS) at a flow rate of 0.5 mL/min at 22°C.
The elution profiles were monitored at an absorbance wavelength of 215 nm. The
CCSL (SEQ ID NO: 1), (VSSLESK)₆ (SEQ ID NO: 9), IL2-like Helix (SEQ ID
10   NO:11) and IL5-like Helix#2 (SEQ ID NO:12) peptides were loaded on the column
in a 100 μL injection at concentrations of 30, 3 and 0.3 μM. Standard globular
proteins were loaded at a concentration of 0.5 mg/mL and included bovine serum
albumin, carbonic anhydrase, cytochrome c and aprotinin (M_r=66, 29, 12.4 and 6.5
kilodaltons respectively). For gel filtration experiments performed in 50%
15   trifluoroethanol (TFE), the PBS was diluted with TFE (1:1, v/v).

        Circular Dichroism spectroscopy. Circular Dichroism (CD) spectra were
measured with a Jasco J-500C spectropolarimeter attached to a Lauda (model RMS)
water bath used to control the cell temperature. The spectropolarimeter was
20   interfaced to a Macintosh computer for data collection and manipulation. All
spectra were measured at 270 to 190 nm using a 0.1 cm cell, 1-nm bandwidth and a
1-s time constant. Four scans were averaged for each spectrum and then corrected
for solvent contributions. Measured rotations were converted to molar mean residue
ellipticity [Θ] (degrees cm^2dmol^{-1}) (Schmid, F.X., in Protein Structure a Practical
25   Approach, Creighton, T.E. (ed), IRL Press, Oxford, p. 283 (1989)). All spectra
were recorded with a cell temperature of 25°C unless otherwise stated. All
concentrations of stock peptide solutions were determined by amino acid analysis.
        To test the effect of the α-helix inducing solvent trifluoroethanol on α-
helical content of the CCSL (SEQ ID NO: 1), IL2-like Helix (SEQ ID NO:11) and
30   IL5-like Helix#2 (SEQ ID NO:12) peptides, CD spectra were recorded for 20 μM
solutions of the various peptides in PBS alone and in a 50% TFE/PBS buffer,
generated by diluting the PBS with TFE (1:1, v/v).
        To determine the effects of concentration of the CCSL peptide (SEQ ID
NO: 1) on α-helical content CD spectra were measured for the peptide at
35   concentrations of 0.2, 1, 10, 100, and 500 μM in PBS.
        To test the effect of pH on the α-helical content of the CCSL peptide (SEQ
ID NO: 1) CD spectra were recorded for a 20 μM solution and were measured from
pH 0.6 to pH 12.5. Stock peptide solutions were prepared with 150 mM sodium

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chloride in dilute phosphoric acid, 20 mM phosphate and in dilute sodium
hydroxide. The desired pH was generated by mixing the appropriate buffered
peptide solutions. The peptide samples were incubated at the desired pH for 1 hour
before taking the CD spectra.

5   To record the thermal denaturation profile for the CCSL peptide (SEQ ID
NO: 1) CD spectra were measured on a 20 μM concentration of the peptide in PBS
in a temperature range of 5°C to 80°C. The sample cell temperature was raised at 5°
C increments and incubated for 15 minutes at each temperature before taking the
CD spectrum.

10   The urea denaturation studies were carried out by preparing CCSL peptide
(SEQ ID NO: 1) stock solutions at a concentration of 20 μM in PBS alone, and with
6 M urea in PBS. Different ratios of the PBS and 6 M urea solutions were mixed to
give the appropriate final urea concentration for the CD measurements. Mixed
samples were incubated for 1 hour before taking the CD spectra.

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α-Helical Content. The CCSL peptide (SEQ ID NO: 1) is highly soluble in
water and PBS which is the first indication that it is assuming a folded conformation
with prevents the exposure of the hydrophobic leucine and valine residues to
solvent. In PBS the CCSL peptide (SEQ ID NO: 1) exhibits a circular dichroism
20   spectrum which is typical of an α-helix with high molar ellipticity minima values at
222 (n-π*) and 208 nm (π-π*), indicating high α-helical content (Figure 3). A
maximum ellipticity signal is also present around 193 nm, however, due to chloride
ions in the buffer and limitations in the spectropolarimeter, values below 192 nm are
deemed unreliable and not shown. The ellipticity minimum at 222 nm which is
25   responsive to α-helical content (Chen et al., Biochem., 13:3350-3359 (1974))
remains unchanged upon the addition of the helix inducing solvent trifluoroethanol
(TFE, 50%) (Figure 3). TFE is considered to be a non interacting solvent that
induces helicity in a single-chain potentially α-helical polypeptide (Nelson and
Kallenbach, Proteins: Structure, Function and Genetics, 1:211-217 (1986)). The
30   less polar solvent induces and stabilizes α-helical structure in a peptide but not
indiscriminately (Hodges et al., Peptide Res., 3:123-137 (1990)). That is, regions
that have a high tendency to adopt an α-helical conformation become more α-
helical in TFE, but regions that are helix-disfavoring are not induced to become α-
helical (Hodges, et al., supra; Nelson and Kallenbach, supra; Nelson and
35   Kallenbach, Biochem., 28:5256-5261 1989)). The inability of TFE to increase the
helical content of the CCSL peptide (SEQ ID NO: 1) indicates that under these
conditions (i.e., pH 7.4, 25°C) the peptide is near its maximum α-helical potential.
The theoretical helicity of the 56 residue polypeptide calculated from the observed

ellipticity at 222 nm (-28,000 deg cm^{2}dmol^{-1}) is estimated to be about 75% (Chen et al., *supra*). Similar results were observed for the IL2-like Helix (SEQ ID NO:11) and IL5-like Helix#2 (SEQ ID NO:12) peptides as well.

5   The addition of TFE to the aqueous buffer does cause a shift and increase in the ellipticity minima around 208 nm for the CCSL peptide (SEQ ID NO: 1) (Figure 3). The π-π* excitation band around 208 nm polarizes parallel to the helix axis and is sensitive to whether the α-helix is single-stranded or is an interacting helix as in the case of the two stranded coiled-coil (Zhou et al., J. Biol. Chem., 267:2664-2670 (1992)). A decrease and red shift in ellipticity minima has been

10   shown to correspond with conversion of a rigid single stranded α-helix to an α-helical coiled-coil structure (Cooper and Woody, Biopolymers, 30:657-676 (1990); Zhou et al., *supra*). Previous studies on parallel coiled-coil peptides report a molar ellipticity ratio at 222 and 208 nm ($[\Theta]_{222}/[\Theta]_{208}$) of about 1.03 for a two-stranded α-helical coiled-coil in aqueous buffer solutions (Lau et al., J. Biol. Chem.,

15   259:13253-13261 (1984); Hodges et al., *supra*; Zhou et al., *supra*) and about 0.86 for a single-stranded α-helix in the presence of TFE (Zhou et al., Biochem., 31:5739-5746 (1992)). For the CCSL peptide in PBS at 25°C the magnitude of the $[\Theta]_{222}/[\Theta]_{208}$ ratio is 1.02 suggesting the α-helices are stabilized in a coiled-coil conformation. In 50% TFE the ellipticity minima shifts to 205 nm and the $[\Theta]_{222}/[$

20      $\Theta]_{208}$ ratio changes to 0.90 indicating single-stranded α-helices. The change in the $[\Theta]_{222}/[\Theta]_{208}$ ratio and the red shift of the ellipticity minima around 208 nm indicate that in PBS the peptide assumes a coiled-coil structure.

CD spectra are obtained at varying concentrations of the CCSL peptide (SEQ ID NO: 1) to determine if the α-helical content of the peptide is dependent on

25   concentration. Peptides with α-helical structures that are dependent on dimerization or oligomerization show a loss of α-helical content as the peptide concentration is decreased (Zhou et al., J. Biol. Chem., 267:2664-2670 (1992); Kaumaya et al., Biochem., 29:12-23 (1990)). Within experimental error the CCSL peptide (SEQ ID NO: 1) shows a similar mean residue ellipticity minima value at 222 nm and no

30   significant difference in the $[\Theta]_{222}/[\Theta]_{208}$ ratio over a 2,500 fold peptide concentration range (500 µM) (Figure 4). These results indicate the α-helical content of the CCSL peptide (SEQ ID NO: 1) is independent of concentration and, therefore, suggest that the α-helices are stabilized through the formation of an intramolecular coiled-coil.

35

Size. Gel filtration chromatography was performed on the CCSL peptide (SEQ ID NO: 1) along with the peptide, (VSSLESK)$_6$, (SEQ ID NO: 9) which is a 42 residue polypeptide which forms an intermolecular homodimer coiled-coil. The

CCSL peptide (SEQ ID NO: 1) elutes from the gel filtration column as a single peak
and at the same position whether the peptide is loaded at a concentration of 30, 3
and 0.3 μM (Figure 5: A, B and C, respectively). With the column calibrated with
standard globular proteins the elution size corresponds to an apparent molecular

5   weight of 6,500 daltons which is close to the calculated monomeric molecular
weight of 5,789 daltons. The slightly larger size determined by gel filtration is
consistent with results seen for other coiled-coil peptides. In benign medium, α-
helical coiled-coils are rod-like in shape and elute more rapidly from a gel filtration
column than a globular molecule of identical molecular weight (Hodges et al., J.

10   Biol. Chem., 256:1214-1224 (1981)). The results that the CCSL peptide (SEQ ID
NO: 1) elutes as a single peak at an apparent molecular weight of 6,500 daltons
independent of starting peptide concentration suggest that it is a monomer in
solution. Similarly, the IL2-like Helix (SEQ ID NO:11) and IL5-like Helix#2 (SEQ
ID NO:12) peptides are monomers in solution.

15   In contrast to the elution profiles of the CCSL peptide (SEQ ID NO: 1), the
(VSSLESK)$_6$ (SEQ ID NO: 9) peptide eluted from the gel filtration column as two
peaks and the proportions of the peaks changed as the concentration of peptide was
lowered (Figure 5: A, B and C). The faster eluting peak was predominate when the
peptide was loaded at a concentration of 30 μM but as the peptide starting

20   concentration was decreased to 0.3 μM, the amount of this peak decreased and the
amount of the slower eluting peak increased to become the dominant peak. These
observations suggest that the faster eluting peak corresponding to an apparent
molecular weight of 10,000 daltons is a dimer of the 42 residue (VSSLESK)$_6$ (SEQ
ID NO: 9) peptide which has a calculated molecular weight of 8,770 daltons. The

25   slower eluting peak represents the monomeric form of the peptide which is in
equilibrium with the dimeric state and therefore increases in proportion to the
dimeric peak as the peptide concentration is lowered. This behavior of the
(VSSLESK)$_6$ (SEQ ID NO: 9) peptide at different concentrations during gel
filtration is consistent with the model that the peptide forms a dimer in solution.

30   These results support the evidence that the elution position and concentration
independence of the CCSL peptide (SEQ ID NO: 1) under identical gel filtration
conditions suggests that in solution it is a monomer.

                Both the CCSL and (VSSLESK)$_6$ (SEQ ID NO: 9) peptides (SEQ ID
NO: 1) elute as a single peak from the gel filtration column with an elution buffer

35   containing 50% of the helix inducing solvent TFE (Figure 5D). The (VSSLESK)$_6$
(SEQ ID NO: 9) peptide elutes at the position seen for the monomeric peptide
eluting in PBS (Figure 5D). There is no indication of a dimeric species present
suggesting that the TFE is stabilizing the single stranded α-helical monomer. In

TFE the CCSL peptide (SEQ ID NO: 1) elutes at an apparent molecular weight of 11,000 daltons which is much larger than the apparent molecular weight determined in PBS (6,500 daltons). These results suggest that TFE stabilizes the CCSL peptide (SEQ ID NO: 1) in an extended single-stranded α-helical conformation, which

5   would appear larger to the gel filtration matrix and elute faster from the column. In TFE the CCSL peptide (SEQ ID NO: 1) elutes faster than the (VSSLESK)$_6$ (SEQ ID NO: 9) peptide which is consistent with it being an extended 56 residue peptide verses the 42 residue α-helix of (VSSLESK)$_6$. Together, the gel filtration experiments performed in PBS and 50% TFE suggest that in PBS the CCSL peptide

10   is folding into a compact monomeric structure.

            Salt Concentration and pH Effects on the Coiled-Coil Structure. The CCSL peptide (SEQ ID NO: 1) dissolved in 20 mM phosphate, pH 7.4, shows a 2 and 5% increase in α-helical content upon the addition of 150 mM and 1M sodium

15   chloride, respectively. This increased helical content with increasing ionic strength is consistent with other coiled-coil peptides and can simply be explained by the increased strength of the hydrophobic interactions in a more polar medium (Lau et al., *supra*); Mo et al., Biopolymers, 30:921-927 (1990)).

            The effect of pH on the CCSL peptide (SEQ ID NO: 1) conformation is

20   shown by analyzing the CD spectra of the peptide at a variety of pHs (Figure 6E). The pH profile shows a non-Bell shaped curve in going from pH 0.6 to pH 12.5 (Figure 6F). The α-helical content dramatically decreases in going from pH 9.3 to pH 12.5. This effect has been reported for a number of coiled-coil peptides. Interestingly the α-helical content drops only slightly in going from the maximum at

25   pH 9.3 to pH 4 and appears to increase slightly upon going to lower pH. In the pH 2-8 range, one would expect that the elimination of charge effects (salt bridges and ion-dipole interactions) due to the protonation of the acidic side chains would result in decreased stability of the helix (Chakrabartty et al., J. Biol. Chem., 264:11307-11312 (1989). However, this increase in stability at low pH has been reported for a

30   number of natural and model coiled-coil peptides (Lowery, J. Biol. Chem., 240:2421-2427 (1965); Lau, et al., *supra*; Hodges et al., *supra*; Zhou et al., *supra*; Huyghues-Despointes et al., Protein Sci., 2:80-85 (1993). It is thought that the free energy of interhelix interaction is predominantly hydrophobic and independent of pH (Skolnick and Holtzer, Macromolecules, 18:1549-1559 (1985)). However,

35   several other factors may contribute to the stability of the individual α-helical segments which are pH dependent. The removal of unfavorable short ranged charge-charge interactions, the effect of unfavorable charge-helix dipole interactions, the existence of a singly charged hydrogen bonds at low pH between

glutamate and lysine (Marquesee and Baldwin, Proc. Natl. Acad. Sci., 84, 8898-8902 (1987)), and possible changes in helix propensity of Glu and Asp that occur on protonation (Skolnick and Holtzer, *supra*; Huyghues-Despointes et al., *supra*) may all contribute to the observed pH dependence.

5   The inset in Figure 6B, shows the decrease in the $[\Theta]_{222}/[\Theta]_{208}$ ratio as the temperature is increased from 5°C to 65°C. The $[\Theta]_{222}/[\Theta]_{208}$ ratio is sensitive to whether the α-helix is single-stranded or in the form of a coiled-coil. The winding of the α-helices around each other or pitch of the coiled-coil is dependent on the number of amino acids per turn of the α-helix (Phillips, G. N.,

10   Proteins: Structure, Function and Genetics, 14:425-429 (1992) and varies widely for different proteins (Seo and Cohen, Proteins: Structure, Function and Genetics, 15:223-234 (1993)). The number of residues per turn in a right-handed α-helix has been shown to vary between 3.50 and 3.65 in globular proteins (Cohen and Parry, Proteins: Structure, Function and Genetics, 7:1-15 (1990)). Correspondingly, the

15   inclination of the "apolar stripe" formed by the residues in positions *a* and *d* on the surface of a single α-helix with heptad substructure could vary substantially, and lie anywhere within the range from 0 to 15°C, so that the crossing angle between two helices would be from 0° to 30°C (Cohen and Parry, *supra*). Supercoiling of the CCSL peptide (SEQ ID NO: 1) as determined by the $[\Theta]_{222}/[\Theta]_{208}$ ratio appears to

20   be sensitive to pH, exhibiting a maximum at pH 7 (Figure 6F, inset).

          Temperature and Urea Denaturation. Figure 6A shows the CD spectra changes recorded for the CCSL peptide (SEQ ID NO: 1) in PBS, over the temperature range of 5°C to 80°C. The peptide shows a decrease in α-helical signal

25   upon thermal denaturation reflecting the transition from a native folded state to a more disordered one. The temperature melting profile shows a gentle decrease in the amount of α-helix up to 60°C and then rapid decrease above 65°C (Figure 6B). Above 65°C the peptide precipitates out of solution which suggests that the molecule unfolds. It is thought that exposure of the hydrophobic leucine and valine

30   residues leads to aggregation. Upon cooling to room temperature this precipitate remains. Since the peptide is insoluble at high temperature it is only possible to estimate the Tm (temperature required at which 50% of the peptide is in its unfolded form) to be greater than 65°C. This high Tm indicates that the α-helical structure of the peptide is remarkably stable. Qualitatively, increasing temperature causes a

35   decrease in both the $[\Theta]_{222}/[\Theta]_{208}$ ratio and in α-helical content which is consistent with the model that supercoiling and α-helical content are strongly coupled.

Urea is commonly used to disrupt the structure of α-helical coiled-coil
peptides. The intensity of the CD signal for the CCSL peptide (SEQ ID NO: 1)
decreased as the urea concentration was increased reflecting the loss of α-helical
structure due to urea denaturation (Figure 6C). The urea denaturation profile shows
5   this gradual decrease in helical content with increasing urea concentration (Figure
6D). A concentration of 2.8 M urea is required to reduce the helical content to 50%.
For simplicity the urea denaturation curve was analyzed by assuming a two-state
folding/unfolding transition (Pace, C.N., Meth. Enz., 131:266-280 (1986)), with an
understanding-that-a-more-complex-unfolding-equilibrium-may-exist-(Bracken-et-al.,
10   Biopolymers, 27:1223-1237 (1988)). The inset of Figure 6D shows the free energy
associated with the unfolding of the CCSL peptide (SEQ ID NO: 1) as a function of
urea concentration. The value of the Gibbs free energy in the absence of denaturant
(ΔG_u) is estimated by linear extrapolation to zero urea. The folded structure of the
CCSL peptide (SEQ ID NO: 1) is stabilized, at 25°C, by a ΔG_u of about 2.2 kcal
15   mol^{-1}.

Formation of an Antiparallel Coiled-Coil. The CCSL peptide (SEQ ID
NO: 1) was synthesized with Acm-blocked amino-and carboxy-terminal cysteine
residues. These blocking groups were removed and a disulfide bond was formed
20   between the cysteine residues by utilizing oxidation with iodine. The disulfide form
of the CCSL peptide (SEQ ID NO: 1) eluted from reverse-phase HPLC much faster
that the Acm-blocked peptide. Incubation of the oxidized CCSL peptide (SEQ ID
NO: 1) in the presence of 5 mM DTT resulted in a shift in the elution position of the
reduced peptide back to the time observed for the Acm-blocked peptide. Air
25   oxidation of the reduced peptide at pH 7.4 completely converted the peptide back to
the disulfide form. In these experiments, Ellman's reagent confirmed the presence
of free thiol groups in the reduced but not the oxidized for of the CCSL peptide
(SEQ ID NO: 1).

In PBS, the disulfide form of the CCSL peptide (SEQ ID NO: 1) eluted
30   form the size exclusion column as a single peak at the same position observed for
the Acm-blocked peptide, suggesting it was a folded monomer. By mass spectral
analysis, this peptide gave a molecular mass of 5661 Da (calculated mass = 5645
Da), confirming that the Acm blocking groups had been removed and the peptide
had not formed an intermolecular disulfide. However, the observed mass was 16 Da
35   higher than the expected mass of 5645 Da. This increase in the mass was probably
due to oxidation of the peptide's single methionine to a sulfoxide as the Acm
blocking groups were removed with iodine.

In PBS, the CD spectrum of the disulfide form of the CCSL peptide (SEQ ID NO: 1) exhibited a small increase in α-helical content versus the reduced peptide, with no change in the $[\Theta]_{222}/[\Theta]_{208}$ ratio. These results suggest that the formation of the disulfide bond does not significantly alter the α-helical structure of

5 the CCSL peptide (SEQ ID NO: 1). The oxidized and reduced forms of the peptide showed similar thermal denaturation profiles form 4 to 60°C. However, at higher temperatures, the oxidized form showed an increased stability, which was likely the result of the formation of a disulfide bond between the otherwise open ends of the coiled-coil. The ability to readily form a disulfide bond between the amino- and

10 carboxy-terminal cysteine residues within the CCSL monomer structure is consistent with the model that the α-helices are stabilized in an antiparallel coiled-coil alignment.

RGD Binding Activity. The binding of the CCSL peptide (SEQ ID NO: 1)

15 and the GRGDMP (SEQ ID NO: 8) peptide to purified GPIIbIIIa was determined by the solid-phase receptor binding assay described by (Smith et al., J. Biol. Chem. 265:12267-12271 (1990)). In competition binding experiments with fibrinogen the GRGDMP and CCSL peptides (SEQ ID NO: 1) gave apparent dissociation constants (K_D) of 150 nM and 180 nM, respectively. The high affinity of the CCSL peptide

20 (SEQ ID NO: 1) for the GPIIbIIIa receptor suggests the RGD sequence present in the loop is readily available for binding. The slight decrease in affinity for the CCSL peptide (SEQ ID NO: 1) verses the linear control peptide GRGDMP (SEQ ID NO: 8) may be due to the restricted conformation of the RGD sequence dictated by the CCSL peptide (SEQ ID NO: 1) stem loop structure. Binding of the CCSL

25 peptide to the GPIIbIIIa receptor provides further evidence that the CCSL peptide folds into a structure which both presents and restricts the conformation of the RGD sequence.

The above examples and description fully disclose the present invention, including preferred embodiments thereof. This invention, however, is not limited to

30 the precise embodiments described herein, but encompasses all modifications within the scope of the claims which follow.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Chaiken, Irwin
                 Graddis, Thomas
                 Myszka, David

    (ii) TITLE OF INVENTION: Coiled-Coil Stem Loop Templates

    (iii) NUMBER OF SEQUENCES: 12

    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: SmithKline Beecham Corporation
        (B) STREET: Corporate Patents / P.O. Box 1539
        (C) CITY: King of Prussia
        (D) STATE: PA
        (E) COUNTRY: USA
        (F) ZIP: 19406-0939

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (vii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: 08/076,088
        (B) FILING DATE: June 11, 1993

    (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Sutton, Jeffrey A.
        (B) REGISTRATION NUMBER: 34028
        (C) REFERENCE/DOCKET NUMBER: P50164-1

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: (215) 270-5024
        (B) TELEFAX: (215) 270-5090

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 56 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: both

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

    Cys Ala Ala Leu Glu Ser Glu Val Ser Ala Leu Glu Ser Glu Val Ala

```
      1                  5                    10                    15
```

```
Ser Leu Glu Ser Glu Val Ala Ala Leu Gly Arg Gly Asp Met Pro Leu
                20                  25                  30
```

```
Ala Ala Val Lys Ser Lys Leu Ser Ala Val Lys Ser Lys Leu Ala Ser
            35                  40                  45
```

```
Val Lys Ser Lys Leu Ala Ala Cys
        50                  55
```

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 13 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (ix) FEATURE:
        (A) NAME/KEY: Region
        (B) LOCATION: 1..13
        (D) OTHER INFORMATION: /note= "Corresponds to amino acids
            335-347 of the E2 protein of human
            Papillomavirus-6."

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Ser Asn Cys Leu Lys Cys Phe Arg Tyr Arg Leu Asn Arg
1                5                    10
```

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 56 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: both

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Cys Ala Ala Leu Ser Asn Cys Val Lys Cys Leu Arg Tyr Arg Val Asn
1                5                    10                  15
```

```
Arg Leu Lys Ala Lys Val Ala Ala Leu Gly Arg Gly Asp Met Pro Leu
                20                  25                  30
```

```
Ala Ala Val Glu Ser Glu Leu Ser Ala Val Glu Ser Glu Leu Ala Ser
            35                  40                  45
```

```
Val Glu Ser Glu Leu Ala Ala Cys
        50                  55
```

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:

          (A) LENGTH: 15 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: peptide

     (ix) FEATURE:
          (A) NAME/KEY: Region
          (B) LOCATION: 1..15
          (D) OTHER INFORMATION: /note= "Corresponds to amino acids
              93-107 of human Interleukin 5 (helix D)."

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

     Leu Asp Tyr Leu Gln Glu Phe Leu Gly Val Met Asn Thr Glu Trp
     1   5   10   15

(2) INFORMATION FOR SEQ ID NO:5:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 56 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: both

     (ii) MOLECULE TYPE: peptide

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

     Cys Ala Ala Leu Ala Ser Ala Val Asp Tyr Leu Gln Glu Phe Val Gly
     1   5   10   15

     Val Leu Asn Thr Glu Val Ala Ala Leu Gly Arg Gly Asp Met Pro Leu
                 20   25   30

     Ala Ala Val Lys Ser Lys Leu Ser Ala Val Lys Ser Lys Leu Ala Ser
                 35   40   45

     Val Lys Ser Lys Leu Ala Ala Cys
     50   55

(2) INFORMATION FOR SEQ ID NO:6:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 19 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: peptide

     (ix) FEATURE:
          (A) NAME/KEY: Region
          (B) LOCATION: 1..19
          (D) OTHER INFORMATION: /note= "Corresponds to amino acids
              108-126 of human Interleuken 4 (Helix D)."

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

     Thr Leu Glu Asn Phe Leu Glu Arg Leu Lys Thr Ile Met Arg Glu Lys

```
     1              5                    10                   15
```

Tyr Ser Lys

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 56 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: both

   (ii) MOLECULE TYPE: peptide

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
   Cys Ala Thr Leu Glu Asn Phe Val Glu Arg Leu Lys Thr Ile Val Arg
   1               5                   10                  15

   Glu Leu Tyr Ser Lys Val Ala Ala Leu Gly Arg Gly Asp Met Pro Leu
               20                  25                  30

   Ala Ala Val Lys Ser Lys Leu Ser Ala Val Lys Ser Lys Leu Ala Ser
           35                  40                  45

   Val Lys Ser Lys Leu Ala Ala Cys
       50                  55
```

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 6 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
   Gly Arg Gly Asp Met Pro
   1               5
```

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 42 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: both

   (ii) MOLECULE TYPE: peptide

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
   Val Ser Ser Leu Glu Ser Lys Val Ser Ser Leu Glu Ser Lys Val Ser
   1               5                   10                  15

   Ser Leu Glu Ser Lys Val Ser Ser Leu Glu Ser Lys Val Ser Ser Leu
               20                  25                  30
```

33

```
         Glu Ser Lys Val Ser Ser Leu Glu Ser Lys
                 35                      40
(2)  INFORMATION FOR SEQ ID NO:10:

       (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 7 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: both

      (ii) MOLECULE TYPE: peptide
```

```
      (ix) FEATURE:
            (A) NAME/KEY: Duplication
            (B) LOCATION: 1..7
            (D) OTHER INFORMATION: /label= Repeat2-15times
                   /note= See Detailed Description for additional
information.
```

```
       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

       Xaa Xaa Xaa Xaa Xaa Xaa Xaa
       1                   5
```

```
(2)  INFORMATION FOR SEQ ID NO:11:

       (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 58 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: circular

      (ii) MOLECULE TYPE: peptide
```
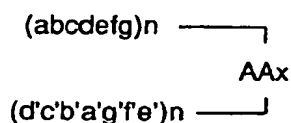
```
       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

       Cys Lys Lys Gln Leu Leu Gln Val Glu His Ala Leu Leu Asp Val Gln
       1               5                   10                  15

       Met Gln Leu Asn Ala Val Asn Asn Ala Leu Gly Arg Gly Asp Met Pro
                   20                  25                  30

       Leu Ala Ala Gln Val Glu Ala Leu Asn Arg Ala Val Thr Ala Leu Gln
                   35                  40                  45

       Ser Gln Val Ser Gln Leu Ala Ala Ala Cys
                   50                  55
```

```
(2)  INFORMATION FOR SEQ ID NO:12:

       (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 58 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: circular

      (ii) MOLECULE TYPE: peptide
```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Thr Ser Ala Lys Leu Lys Glu Val Leu Ala Lys Leu Ser Thr Val Arg
1             5               10              15

Thr Lys Leu Ile Ala Val Glu Thr Lys Leu Gly Cys Gly Asp Met Pro
            20              25              30

Leu Ala Ala Glu Val Ala Ala Leu Asn Gln Glu Val Asp Ala Leu Gln
        35              40              45

Glu Glu Val Ala Ala Leu Asn Thr Glu Ala
    50              55
```

Claims

What is claimed is:

5   1.   A non-naturally occurring two-helix coiled-coil stem loop template
molecule which comprises a single polypeptide chain comprising a first α-helical
structure in an aqueous environment, a second α-helical structure in an aqueous
environment and a loop segment which connects the carboxyl terminus of said first
α-helical structure to the amino terminus of said second α-helical structure wherein
10   said first and second α-helical structures are arranged longitudinally in an
antiparallel orientation such that said first and second helices form an
intramolecular, monomeric, superhelical structure.

2.   The molecule of claim 1 wherein both α-helical structures form
15   heptad repeats $(abcdefg)_n$ (SEQ ID NO: 10) of equal length, of the formula

$$(abcdefg)n \quad \text{---} \quad \rceil$$
$$\text{AAx}$$
$$(d'c'b'a'g'f'e')n \quad \text{---} \quad \rfloor$$

wherein:

when positions a and d' are hydrophobic residues independently selected
20   from the group consisting of alanine, isoleucine, methionine and valine, then
positions d and a' are leucine or when positions d and a' are hydrophobic residues
independently selected from the group consisting of alanine, isoleucine, methionine
and valine, then positions a and d' are leucine with the proviso that the a or d
position of the first heptad repeat, which ever occurs first, and the last d' or a'
25   position of the last heptad repeat, whichever occurs last, is any amino acid residue;
e and g and e' and g' are independently selected from the group consisting
of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid,
histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine,
tryptophan, tyrosine, and valine with the proviso that when one of two consecutive
30   heptad e, g, e' or g' positions is so selected from the group of hydrophobic-like
residues consisting of valine, leucine, isoleucine, methionine, phenylalanine and
tryptophan then the other heptad position may not be selected from the same group
of hydrophobic-like residues, and with the additional proviso that each e and e' shall
not both be positively or negatively charged and each g and g' shall not both be
35   positively or negatively charged;

36

b, c, f, b', c' and *f* are any amino acid residue with the proviso that there
cannot be more than one glycine or proline residue per heptad repeat;

n is 2-15 or fractions thereof in-between those integers; and

AAx forms a polypeptide loop segment of 4 to 15 amino acid residues.

5

3.   The molecule of claim 2 wherein the hydrophobic residues of
positions *a* and *d'*, or *d* and *a'*, are the same amino acid selected from the group
consisting of alanine, isoleucine, methionine and valine.

10   4.   The molecule of claim 3 wherein the hydrophobic residues of
positions *a* and *d'*, or *d* and *a'*, are further selected from the group consisting of
alanine, isoleucine and valine.

5.   The molecule of claim 4 wherein the hydrophobic residues of
15   positions *a* and *d'*, or *d* and *a'*, are still further selected from the group consisting of
alanine and valine.

6.   The molecule of claim 5 wherein positions *a* and *d'*, or *d* and *a'*, are
valine.

20

7.   The molecule of claim 2 wherein *e* and *g* and *e'* and *g'* are selected
from the group consisting of alanine, arginine, asparagine, aspartic acid, cysteine,
glutamine, glutamic acid, histidine, lysine, serine, threonine and tyrosine.

25   8.   The molecule of claim 7 wherein *e* and *g* and *e'* and *g'* are further
selected from the group consisting of aspartic acid, glutamic acid, lysine, arginine
and histidine.

9.   The molecule of claim 8 wherein the overall net charge of all *e* and *g*
30   positions of the first α-helical structure is opposite to the overall net charge of all *e'*
and *g'* positions of the second α-helical structure.

10.   The molecule of claim 2 wherein one of the following positions, *b, c*
or *f* is any amino acid and remaining two positions are independently selected from
35   the group consisting of alanine, arginine, asparagine, aspartic acid, cysteine,
glutamine, glutamic acid, lysine, serine, threonine and tyrosine, and one of the
following positions, *b', c'* or *f* is any amino acid and remaining two positions are
independently selected from the group consisting of alanine, arginine, asparagine,

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aspartic acid, cysteine, glutamine, glutamic acid, lysine, serine, threonine and
tyrosine.

11.   The molecule of claim 10 wherein said remaining positions are
further selected from the group consisting of alanine, serine and threonine.

12.   The molecule of claim 2 wherein *b, c, f, b', c'* and *f* are further
selected from the group consisting of alanine, arginine, asparagine, aspartic acid,
cysteine, glutamine, glutamic acid, lysine, serine, threonine and tyrosine.

13.   The molecule of claim 2 wherein n is 3 - 10 or fractions thereof in
between those integers.

14.   The molecule of claim 13 wherein n is 3 - 7 or fractions thereof in
between those integers.

15.   The molecule of claim 2 wherein the loop segment comprises from
about 5 to 10 residues.

16.   The molecule of claim 15 wherein the first and last residue of the
loop segment are selected from the group consisting of glycine and proline.

17.   A pharmaceutical composition comprising the coiled-coil stem loop
template molecule of claim 2.

18.   An antibody directed to the molecule of claim 1.

19.   The antibody of claim 18 which is a monoclonal antibody.

20.   An isolated DNA molecule which encodes the coiled-coil stem loop
template molecule of claim 2.

21.   A vector comprising the recombinant DNA molecule of claim 20.

22.   The vector according to claim 21 which is a plasmid.

23.   The vector according to claim 21 which is a phage.

24.   The phage of claim 23 which displays a coiled-coil stem loop template molecule as a filamentous phage surface protein.

25.   A process to elicit antibodies directed to small peptide molecules which comprises inoculation of a selected mammal with the coiled-coil stem loop molecule of claim 1 wherein the sequence of said small peptide molecule is found in the loop region of said coiled-coil stem loop molecule.

26.   A method to produce helical recognition mimics which comprises synthesis of the coiled-coil stem loop molecule of claim 2 wherein the recognition sequences are incorporated into the b, c. f, b', c' and f' positions of the heptad repeat.

27.   A method to produce helical recognition mimics which comprises synthesis of the coiled-coil stem loop molecule of claim 2 wherein the recognition sequences are incorporated into the b, c. e, f, g, b', c', e', f' and g' positions of the heptad repeat.

28.   A polypeptide produced by the process of claim 27.

29.   A method to antagonize binding of a ligand to its receptor wherein the ligand or receptor has an α-helical structure involved in ligand-receptor binding, which comprises binding to said ligand or receptor a coiled-coil stem loop template molecule that mimics the conformational elements of said α-helical structure.
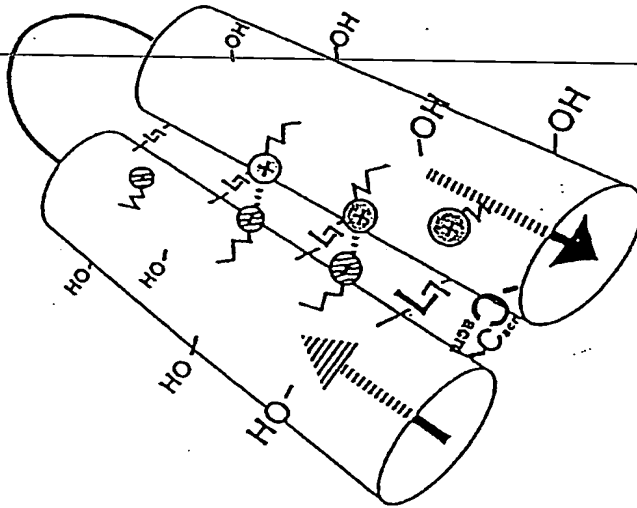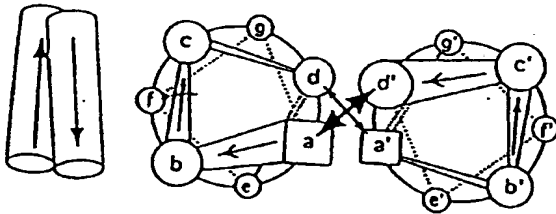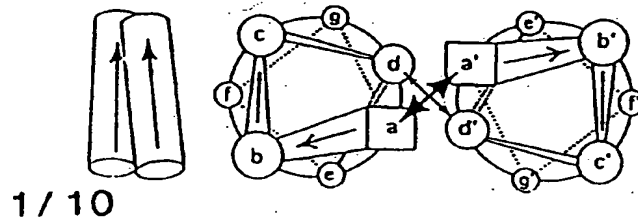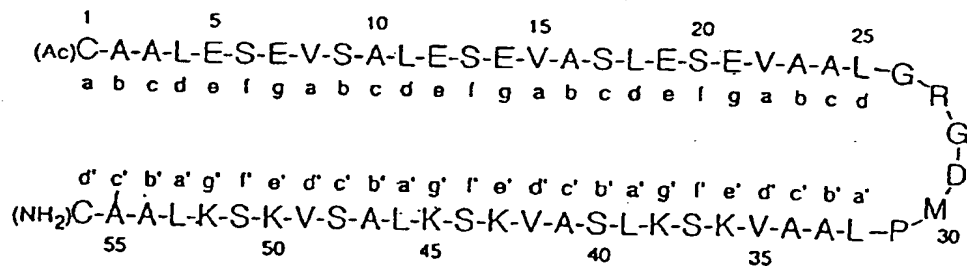
FIGURE 1A

FIGURE 1B

FIGURE 1C

FIGURE 2A

CCSL
Template

FIGURE 2B

HPV-6 DNA
Binding Helix

335-S-N-C-L-K-C-F-R-Y-R-L-N-R-347

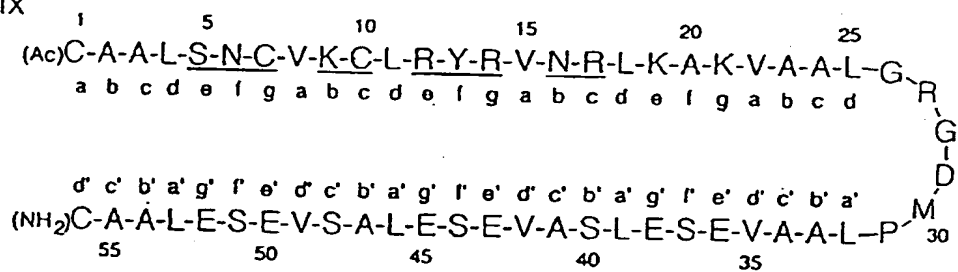2 / 10

FIGURE 2C

IL5 Helix D:

93-L-D-Y-L-Q-E-F-L-G-V-M-N-T-E-W-107

(Ac)C-A-A-L-A-S-A-V-D-Y-L-Q-E-F-V-G-V-L-N-T-E-V-A-A-L—G
  a b c d e f g a b c d e f g a b c d e f g a b c d   R
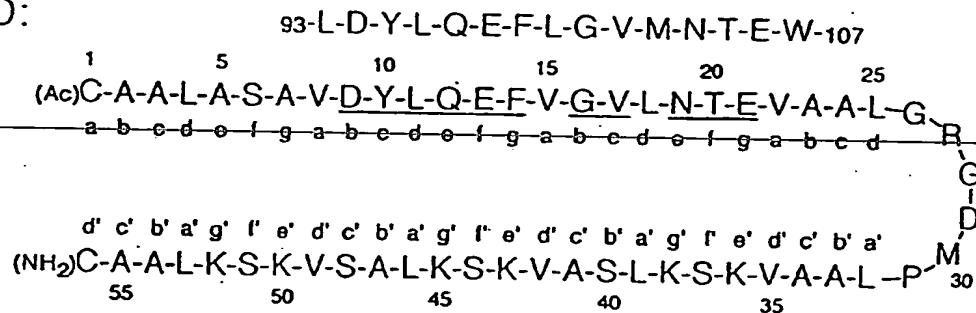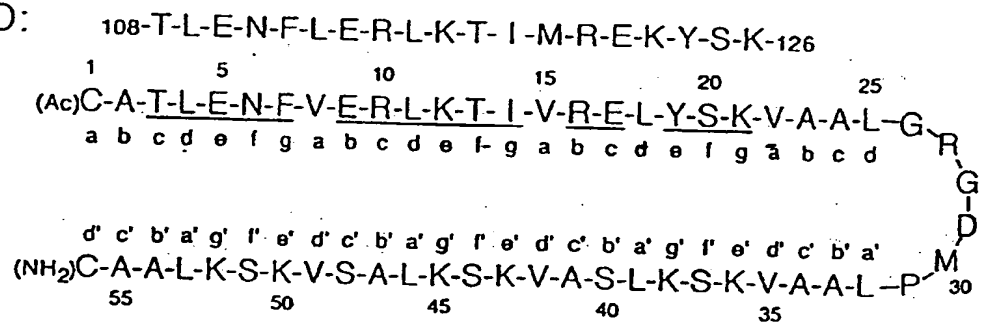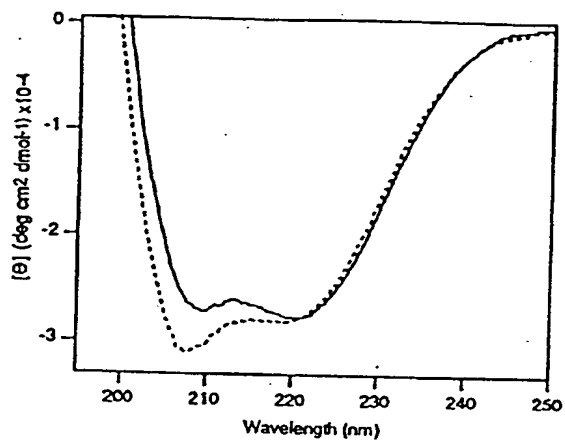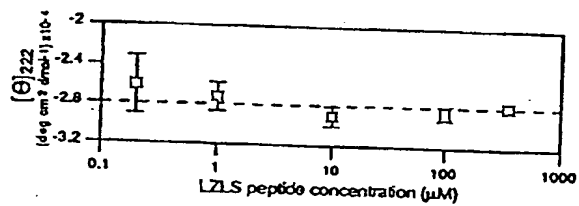   G
                                                                                D
d' c' b' a' g' f' e' d' c' b' a' g' f' e' d' c' b' a' g' f' e' d' c' b' a'   M
(NH₂)C-A-A-L-K-S-K-V-S-A-L-K-S-K-V-A-S-L-K-S-K-V-A-A-L—P 30
     55   50   45   40   35

FIGURE 2D

IL4 Helix D:

108-T-L-E-N-F-L-E-R-L-K-T- I-M-R-E-K-Y-S-K-126

(Ac)C-A-T-L-E-N-F-V-E-R-L-K-T- I-V-R-E-L-Y-S-K-V-A-A-L—G
   a b c d e f g a b c d e f g a b c d e f g a b c d   R
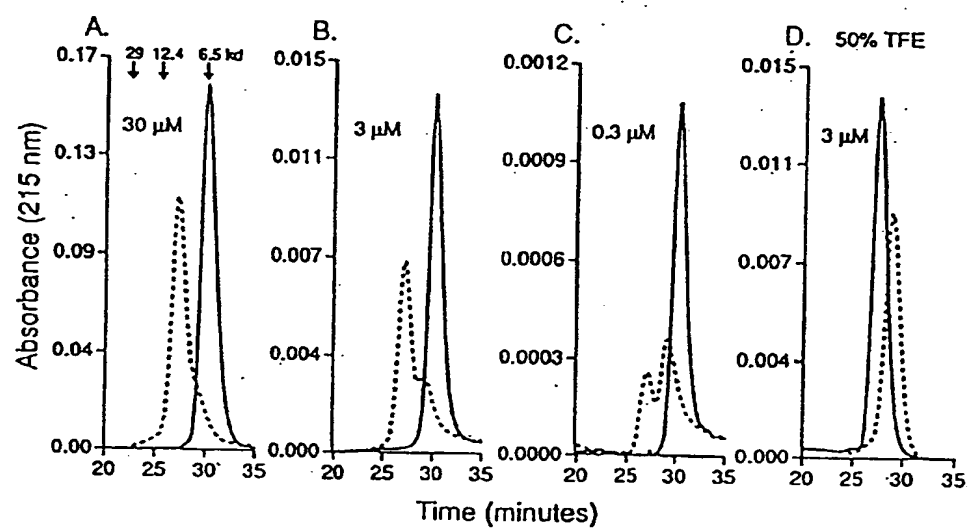   G
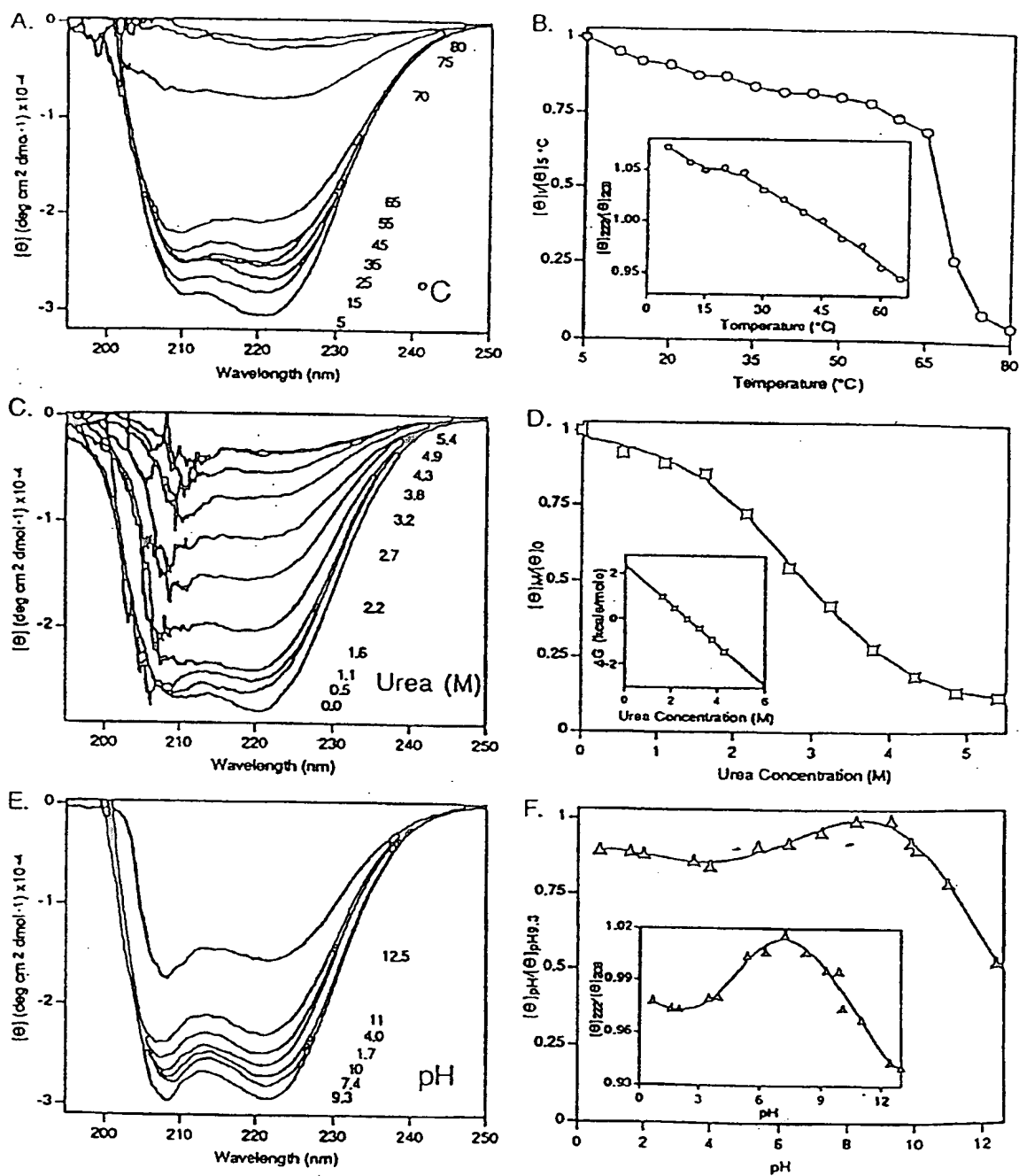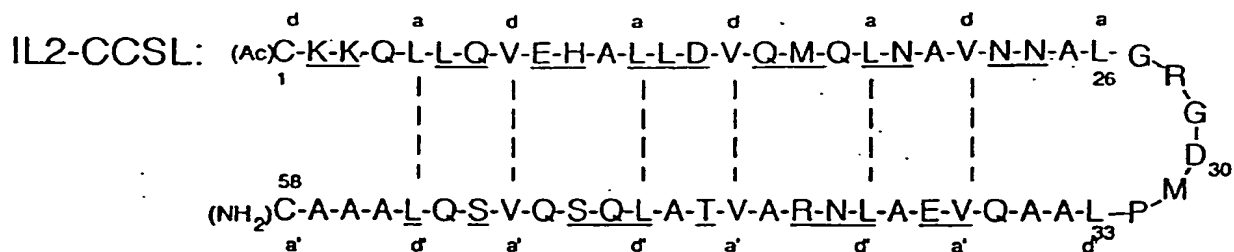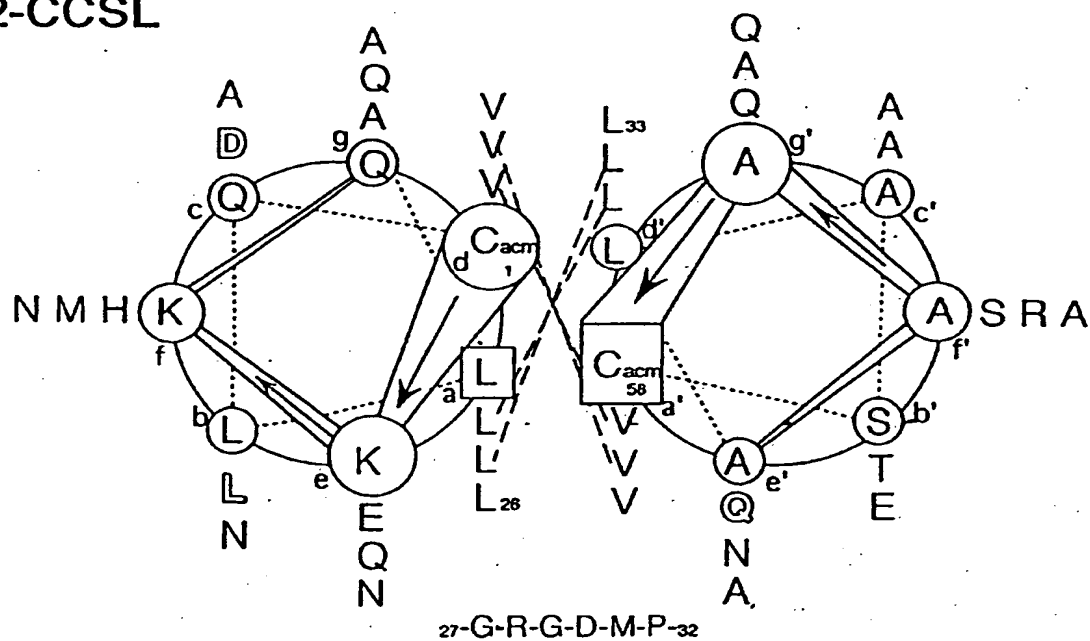   D
d' c' b' a' g' f' e' d' c' b' a' g' f' e' d' c' b' a' g' f' e' d' c' b' a'   M
(NH₂)C-A-A-L-K-S-K-V-S-A-L-K-S-K-V-A-S-L-K-S-K-V-A-A-L—P 30
     55   50   45   40   35

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FIGURE 3

FIGURE 4

4 / 10

FIGURES 5A - 5D

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FIGURES 6A - 6F

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FIGURE 7A

IL2-CCSL: (Ac)C-K-K-Q-L-L-Q-V-E-H-A-L-L-D-V-Q-M-Q-L-N-A-V-N-N-A-L-G

(NH₂)C-A-A-A-L-Q-S-V-Q-S-Q-L-A-T-V-A-R-N-L-A-E-V-Q-A-A-L-P

Dashed lines indicate the hydrophobic coiled coil interface interactions.

Underlined amino acids are conserved from the native IL-2 sequence.
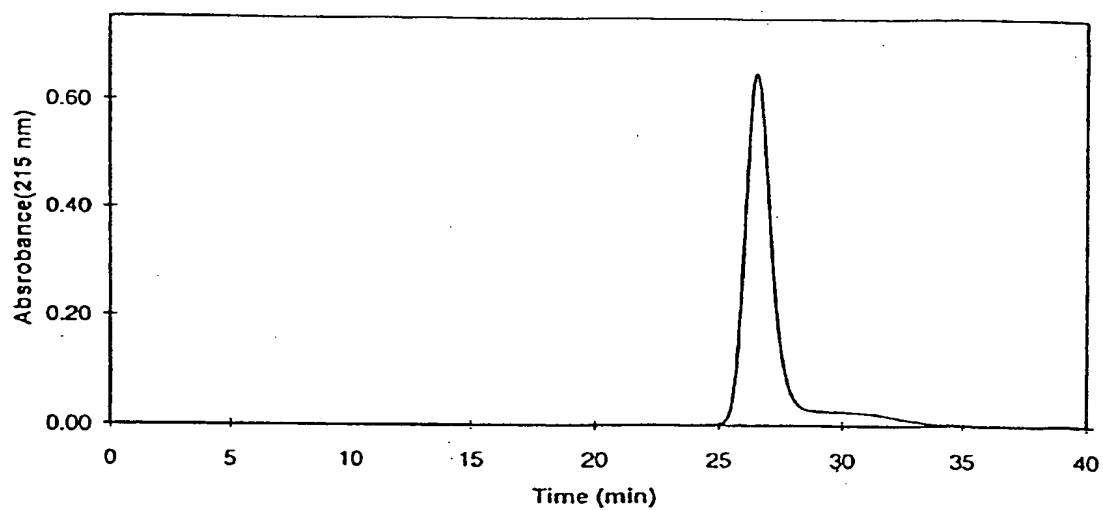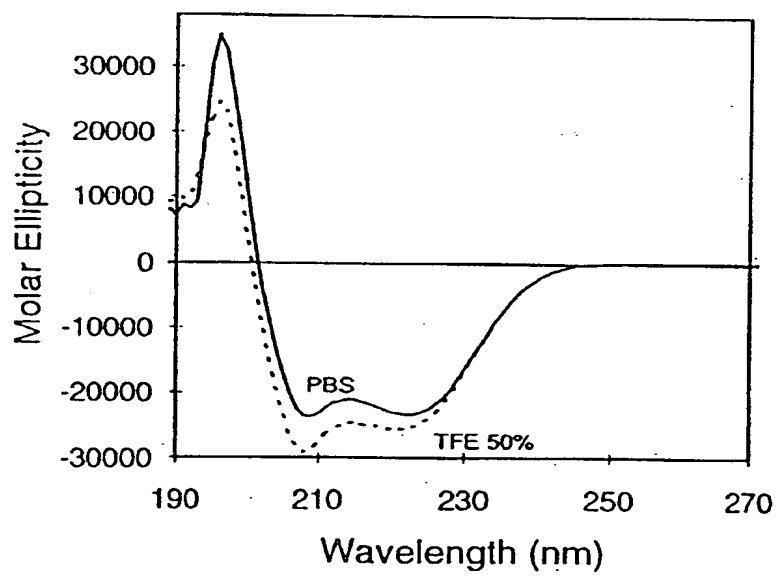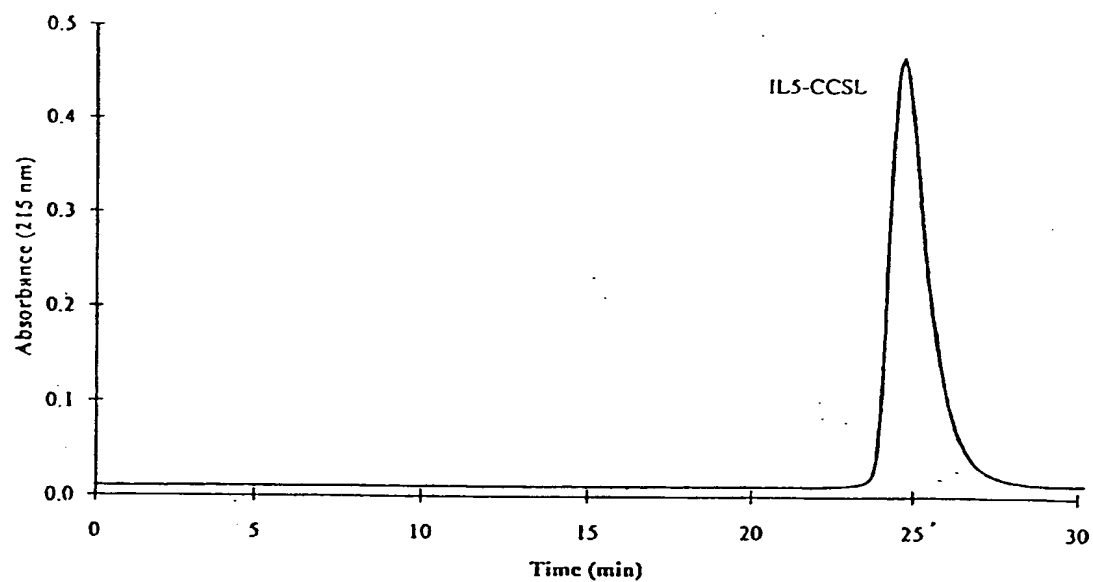
FIGURE 7B

IL2-CCSL

27-G-R-G-D-M-P-32

IL-2CCSL-4

FIGURE 8B

IL2-CCSL#4
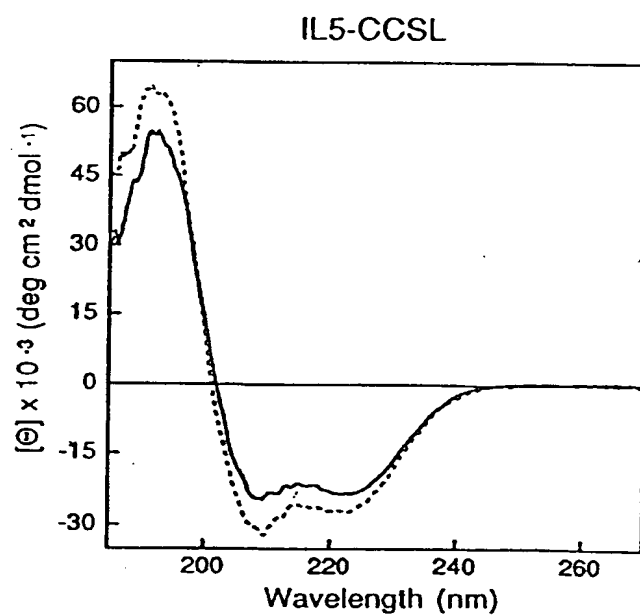
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FIGURE 9A

IL5-CCSL: ₁ T-S-A-K-L-K-E-V-L-A-K-L-S-T-V-R-T-K-L-I-A-V-E-T-K-L-G

with positions labeled d, a, d, a, d, a, d, a (26) across the top

           G
           C
           G
           D ₃₀
           M

58 A-E-T-N-L-A-A-V-E-E-Q-L-A-D-V-E-Q-N-L-A-A-V-E-A-A-L-P

with positions labeled a', d', a', d', a', d', a', d' (33) across the bottom

FIGURE 9B

IL5-CCSL

27-G-C-G-D-M-P-32

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FIGURE 10A

FIGURE 10B

IL5-CCSL

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A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 1/04, 7/04, 15/12; A61K 37/02

US CL :530/300, 333, 350, 403; 514/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 333, 350, 403; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CA, BIOSIS, JICST-E, INPADOC, MEDLINE search terms: coiled coil, peptide, protein, poylpeptide, loop, antiparallel

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 267, Number 9, issued 25 March 1992, Kaumaya et al, "Design and Immunological Properties of Topographic Immunogenic Determinants of a Protein Antigen (LDH-C₄) as Vaccines", pages 6338-6346, see entire document.	1, 17, 28
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Y		2-16, 26, 27
X,P	BIOCHEMISTRY, Volume 33, Number 9, issued 08 March 1994, Myszka et al, "Design and Characterization of an Intramolecular Antiparallel Coiled Coil Peptide", pages 2363-2372, see Figure 1, page 2364.	1-17, 26-28
Y	PROTEINS, Volume 7, Number 1, issued 1990, Cohen et al, "α-Helical Coiled Coils and Bundles: How to Design an α-Helical Protein", pages 1-15, see entire document.	1-17, 26-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 AUGUST 1994	AUG 09 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	STEPHEN WALSH
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOSCIENCE REPORTS, Volume 2, issued 1982, Parry, "Coiled-coils in α-helix-containing proteins: analysis of the residue types within the heptad repeat and the use of these data in the prediction of coiled-coils in other proteins", pages 1017-1024, see pages 1021-1023.	1-17, 26-28
Y	BIOCHEMISTRY, Volume 29, Number 1, issued January 1990, Kaumaya et al, "Synthesis and Biophysical Characterization of Engineered Topographic Immunogenic Determinants with αα Topology", pages 13-23, see entire document.	1-17, 26-28

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such
   an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-17, 26-28

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I.   Claims 1-17 and 26-28, drawn to a non-naturally occurring two-helix coiled-coil stem loop template molecule,
        a pharmaceutical composition, and a method of producing a molecule, classified in class 514, subclass 2.

II.   Claims 18, 19 and 25, drawn to an antibody and a process to elicit antibodies, classified in class 530, subclass
        387.1

III.   Claims 20-24, drawn to an isolated DNA molecule, a vector and a phage, classified in class 435, subclass
        320.1.

IV.   Claim 29, drawn to a method to antagonize binding of a ligand to its receptor, classified in class 435, subclass
        7.1.

        The compositions of I-III are materially distinct compositions of matter, each from the other, and the method
of I does not use or produce either of the compositions of II or III. The method of IV as claimed does not use or
produce any of the compositions of I-III. Accordingly, the claims are not so linked by a special technical feature within
the meaning of PCT Rule 13.2 so as to form a single general inventive concept.